

Parasite-host interaction of the  
protoplast isolates of Entomophthora  
egressa with the eastern hemlock looper  
and the eastern spruce budworm

By



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### Abstract

Hemocytes of the insects Labdina fiscellaria fiscellaria and Choristoneura fumiferana did not adhere to the protoplasts of the fungus Entomophthora egressa. Hemocyte reaction for both insect species to test-particles was not suppressed by the protoplasts. The spherule cells of L. fiscellaria fiscellaria adhered to the spherical hyphal bodies and hyphae of E. egressa. The granular cells of C. fumiferana adhered to the hyphae of E. egressa. Protoplasts exposed to papain were attacked by the granular cells of C. fumiferana.

Spent growth medium of both protoplast isolates produced paralysis when injected into C. fumiferana larvae. Evidence suggests that heat-stable proteins may be involved.

Protoplast isolates showed differences in the growth rates and regeneration sequences using coagulated egg yolk medium, a highly modified version of Grace's insect tissue culture medium (MGM) and modifications of MGM and in the presence of CO<sub>2</sub>. The isolates also differed in the changes that they induced in MGM composition during protoplast growth and in the rates of glucose utilization and protein secretion.



The serum of C. fumiferana larvae contained protein(s) which we believe adhere to the cell membranes of the protoplasts of E. egressa. Evidence is presented for hemocyte-plasma interaction in the presence of protoplasts. Components in the larval serum were found to influence protoplast growth patterns. The possibility of antiprotoplast serum activity is presented. Melanin, toxic levels of ninhydrin-positive compounds and antiprotoplast proteins may have been involved in this activity.

The granular cells of L. fiscellaria fiscellaria and C. fumiferana adhered to the hyphae of Rhizopus nigricans. Spores of Absidia repens and the bacteria Escherichia coli and Bacillus cereus adhered to the granular cells of both species of insects. The granular cells and plasmatocytes of C. fumiferana were capable of phagocytosing B. cereus. Adhesion of A. repens spores to C. fumiferana granular cells was stimulated by N-acetylglucosamine and glucosamine, moderately reduced by D-fucose, D-arabinose, D-mannose, D-galatose and sucrose and mildly reduced by D-glucose, D-fructose and trehalose. There was no evidence of humoral opsonins in larval hemolymph favoring test-particle-hemocyte interaction. Granular cells of C. fumiferana exposed to papain had reduced affinities for A. repens spores.

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List of Abbreviations

ADHC	-	Absolute differential hemocyte counts
AHC	-	Absolute hemocyte counts
DHC	-	Differential hemocyte counts
EDTA	-	Ethylenediaminetetraacetic acid
FM	-	Medium based on female larval serum ninhydrin-positive compounds
GlcA	-	Glucosamine
GlcNAc	-	N-acetylglucosamine
GT	-	Generation time
I458	-	Isolate 458 from <u>Lambdina fiscellaria fiscellaria</u>
I521	-	Isolate 521 from <u>Choristoneura fumiferana</u>
Mcct	-	Microcapacity centrifuge tube technique
MES	-	2-(N-morpholino)ethanesulfonic acid
MGM	-	Modified Grace's insect tissue culture medium
ML	-	Monolayer
MN	-	Medium based on male larval serum ninhydrin-positive compounds
NPC	-	Ninhydrin-positive compounds
PC	-	Pooled controls
PCHL	-	Hemolymph plus <u>Paramecium caudatum</u>
PE	-	Protoplasmic extensions
PH	-	Hemocytes plus <u>P. caudatum</u>
PS	-	Serum plus <u>P. caudatum</u>
PTU	-	Phenylthiourea
Rms	-	Relative mobilities
Shb	-	Spherical hyphal bodies



TCA - Trichloroacetic acid  
THC - Total hemocyte counts  
TU - Trypsin unit



## Introduction

The fungus Entomophthora egressa MacLeod and Tyrrell contributed to the collapse of infestations of the eastern hemlock looper, Lambdina fiscellaria fiscellaria (Guenée) in Newfoundland forests (Otvos et al. 1973). Vandenberg and Soper (1978) reported that E. egressa was the prevalent species of Entomophthora infecting the eastern spruce budworm, Choristoneura fumiferana (Clemens).

The wall-free protoplast stage of E. egressa was first produced in culture by Tyrrell and MacLeod (1972) when conidia were germinated in Grace's modified insect tissue culture medium (Grace 1962, 1966). Injection of the protoplasts into Malacosoma disstria Hübner, Orygia leucostigma L., C. fumiferana and L. fiscellaria fiscellaria resulted in the death of the insects and the production of conidia (Tyrrell and MacLeod 1972). The hemocoel of the insects provided a compatible environment for the growth and development of the protoplast stage.

Tyrrell (1977) sprayed eastern spruce budworm larvae with conidia of E. egressa and found protoplasts in the host hemolymph. Tyrrell's photomicrographs indicated no obvious physical interaction between the protoplasts and the host hemocytes.



Otvos et al. (1973) reported that eastern hemlock looper larvae infected with injected protoplasts of E. egressa produced conidia capable of infecting non-diseased larvae. This suggests that the protoplast stage may be the normal vegetative stage of E. egressa in the eastern hemlock looper. The nature of the parasite-host interaction is unknown. An understanding of the nature of interaction of several protoplast isolates of E. egressa with the hemolymph and its components of the eastern spruce budworm and eastern hemlock looper could aid in the effective application of E. egressa in biological control programs against these insects by adding to our knowledge of parasite infection and host defense mechanisms.



### Literature Review

The main emphasis of previous studies of insect mycoses has been in epizootology, taxonomy, invasion routes and mechanisms, fungal morphogenesis and toxicology (See Ferron 1978). There is an extreme paucity of data on the immediate interaction between host insect hemocytes and fungal pathogens, the reactions of which may determine the success of a fungal insect pathogen.

After penetrating the host cuticle, the fungus encounters the cellular defense reactions of the hemocytes of the insect. Hemocyte defense reactions against entomophagous fungi and other insect pathogens have been briefly discussed by Whitcomb et al. (1974), Ferron (1978) and Ratcliffe and Price (1979).

Depending on the host species, fungal species and its stage of development, four hemocyte defense responses have been described; phagocytosis, encapsulation, humoral encapsulation, and the absence of physical hemocyte activity.

The hemocytes of Talitrus sp. have been reported to phagocytose spores of Oidium sp. (Hermann and Canu 1891). De Bary (1887) described the phagocytosis of hyphae of



Cordyceps militaris (Fries) Link by the "leukocytes" of the Sphingidae moths. This is unusual in that small objects are usually phagocytosed by insect hemocytes (Ratcliffe and Rowley 1979) and large objects are generally encapsulated (Salt 1970).

Speare (1920) reported that phagocytosis conferred significant resistance to fungal insect pathogens. In order to kill larvae of Bombyx mori L. and a species of Lachnosterna using Sorospora uvella (Krassiltschik) Giard, to which the larvae were normally resistant, it was necessary to inject sufficient numbers of spores into the hemolymph to exceed the phagocytic capacity of the hemocytes.

Hemocytes in chilled, diapausing pupae of Hyalophora cecropia L. readily engulfed the conidiospores of mildly pathogenic Aspergillus niger v. Teigh but spores of highly pathogenic Aspergillus flavus Link were poorly phagocytosed (Sussman 1952). It is generally assumed that the degree of virulence of the pathogen is related to the degree of host resistance (Ferron 1978). Kawakami (1965) described the active phagocytosis of the conidia and hyphal bodies of two muscardine-fungi, Paecilomyces fumoso-roseus (Wize) (= Isaria fumosorosea Wize) and Harziella entomophila Ishiwata and Miyake by insect hemocytes.



Encapsulative responses of the hemocytes of the parasitoid Exeristes comstockii (Cresson) to the yeast Torula nigra (Marpmann) have been described by Bucher and Bracken (1966). The capsule consisted of outer layers of intact hemocytes surrounding an inner layer of necrotic hemocytes effectively walling off the yeast from the host. Vey (1968) reported that the plasmatocytes of Galleria mellonella L. encapsulated Mucor hiemalis Wehmer after the fungus had entered the hemocoel via wounds. Encapsulation was ineffective because of the rapid growth of the hyphae and the toxins produced. In contrast, Mohamed et al. (1978) believed that the hemocytes of Heliothis zea (Boddie) were attacked and broken down by hyphae of Nomureae rileyi Maublanc. Hyphae and conidia of A. niger injected into the hemocoel of G. mellonella were readily encapsulated within 72h (Vey and Vago 1969). This phenomenon has been duplicated in vitro (Vey 1969). The capsules were composed of layers of plasmatocytes connected by intercellular cement. The plasmatocytes nearest the hyphae were partially lysed and the fungal elements were surrounded by melanin. Vey (1971) described the ultrastructural features which were comparable to the capsules described by Grimstone et al. (1967) and Salt (1970) for insects in general.



The hyphal tips of Metarhizium anisopliae (Metsch.) Sorok. and Beauveria bassiana (Bals.) Vuill. were encapsulated by hemocytes in G. mellonella but with no significant effect on the development of the mycoses (Prasertphon and Tanada 1968). Gardner and Noblet (1978) have recorded similar results in Heliothis virescens (Fabricius) with B. bassiana. Moderately effective containment of hyphae of B. bassiana has been reported for capsules in Porthetria dispar (L.) (Wasti and Hartmann 1975). Farques et al. (1976) found that two strains of M. anisopliae tested on both their original hosts, Oryctes rhinoceros L. and Cetonia aurata L., and on the heterogenous host were subjected to similar hemocyte responses, but eventually only the specific pathotype passed through the capsule.

Sweeney (1975) described the ineffective, massive encapsulative response of the hemocytes of Culex fatigans Wiedemann larvae to hyphae of Culicinomyces sp..

Chironomus thummi Meigen larvae with few freely circulating hemocytes form layers of putative melanin around the spores of A. niger and sporangiospores of M. hiemalis injected into the hemocoel. Humoral encapsulation was completely effective against the former species and partially effective against the latter species (Gotz and Vey 1974).



The absence of physical interaction between invading fungi and host hemocytes is rare among the Deuteromycetes. Hemocytes of a Dasyhelea sp. did not adhere to the germ tubes of Culicinomyces (Sweeney 1975). It was speculated that in the proper host there is no hemocyte reaction.

Coelomomyces punctatus Couch is believed to evade the hemocytes of Anopheles quadrimaculatus Say by confusing the hemocytes with portions of cytoplasm pinched off the thallus (Powell 1976).

Seryczynska and Bajan (1975), while not describing physical hemocyte-hyphal contact, reported that the hemocytes of Leptinotarsa decemlineata Say increased in number during the initial 12h following infection with P. fumosoroseus, Paecilomyces farinosus (Dicks) Brown and Smith, and B. bassiana. The hemocyte levels decreased thereafter. The degree of change was related to the type of pathogen.

Hemocyte involvement with representatives of the Entomophthorales has rarely been reported. A survey of the literature has shown that the majority of species of Entomophthora when examined soon after penetrating the hemocoel and during the proliferation of either hyphae or hyphal bodies over a 48-72h period failed to show definitive signs of hemocyte involvement (see Sawyer 1933,



Yen 1962, Hutchison 1962, Yendol and Paschke 1965, Prasertphon and Tanada 1968, Hartmann and Wasti 1976 and Carner 1976). Brobyn and Wilding (1977) reported the absence of physical hemocyte-hyphal body contact for three species of Entomophthora in aphids. Klein and Coppel (1973) reported that encapsulation of Entomophthora tenthredinus Fres. in Diprion similis (Hartig) partially inhibited fungal development.

Dresner (1949) reported that germinating spores of B. bassiana secreted a toxin\* which produced a "knockdown" effect on Musca domestica L. adults by rapid paralysis. Acetone extracts of culture filtrates of Aspergillus ochraceus Wilhelm, Sterigmatocystis japonica Aoki, B. bassiana, Spicaria pracina (Maubli) Aoki, P. farinosus (= Isaria farinosa), P. roseus (= Isaria rosea) Wize, M. anisopliae, A. flavus and Aspergillus oryzae Wehmer were reported to be toxic when injected into B. mori (Kodaira 1954, 1959). Metarhizium anisopliae produced the most toxic agent (Kodaira 1959).

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\* A toxin, as defined by Prasertphon and Tanada (1969), is a metabolite of a microorganism or a microorganism-host interaction which acts directly on and is detrimental to living host cells. A mycotoxin is a toxin produced by a fungus.



Aspergillus flavus var. columnaris Link produced a water-soluble, extracellular metabolite lethal to maggots of M. domestica (Beard and Walton 1965). Gudauska et al. (1966) documented the lethality of aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> from A. flavus to H. virescens. A mixture of these aflatoxins reduced the fecundity of Aedes aegypti L., Drosophila melanogaster Meigen and M. domestica when administered per os at a level of 0.06 ppm (Matsumura and Knight 1967). Aflatoxins are known chemosterilants for Sitophilus oryzae L. (Srinath et al. 1973), Trombidium gigas L. (Sannasi and Amirthavalli 1970) and Anthonomus grandis Boheman (Moore et al. 1978). High mortality from spraying aflatoxin mixtures has been reported for Dendroctonus frontalis Zimmerman (Moore 1971) and Epilachna vigintioctopunctata (F.) (Krishnamoorthy and Saukar Naidu 1971). Beard and Walton (1971) detected a delay in larval development and reduced size of surviving flies of M. domestica exposed to sublethal doses of aflatoxins. This effect was not detected in either G. mellonella or Plodia interpunctella Hubner. Kojiic acid, a metabolite of A. flavus was found to be toxic to M. domestica larvae (Beard and Walton 1971). A reduction in the pupal case size of D. melanogaster with increasing concentration of aflatoxin B<sub>1</sub> has been documented by Lalor et al. (1976). The few flies emerging were weak and soon died.



Llewellyn and Chinnici (1978) have shown that the effects of aflatoxins vary with the strain of test insect. Aflatoxin B<sub>1</sub> differed in the degree of effectiveness on the growth rates, sex ratio and egg viability for different strains of D. melanogaster.

The cyclodepsipeptides, destruxins A and B, isolated from growth media of M. anisopliae are lethal to B. mori and Epilachna sparsa L. upon intrahemocoelomic injection (Kodaira 1960, 1961). Roberts (1964, 1966a, 1966b) isolated toxins from M. anisopliae which induced tetanic paralysis in larvae of G. mellonella and B. mori. Larvae of B. mori naturally infected by M. anisopliae exhibit tetanic paralysis prior to death. Heat-treated hemolymph from these larvae was toxic to G. mellonella when injected into the hemocoel. The toxins produced by the fungus may play an important role in the mycosis (Roberts 1966b). Suzuki et al. (1970) have expanded the types of destruxins to destruxins C and D.

Mosquito larvae of Culex pipiens Wied. died after ingesting spores of M. anisopliae that failed to germinate (Roberts 1970). Crisman (1971) has suggested that the digestion of the walls of the spores by the larval gut enzymes released the fungal toxins.



Histological studies of tissues of elaterid beetle larvae attacked by M. anisopliae showed that the toxins produced progressive degeneration of host tissues, with no appreciable swelling or shrinking of host cells. The primary effect appeared to be on the structural integrity of the membranes of various cellular organelles (Zacharuk 1971). Cordyceps militaris produced cytopathic effects on cells of Aedes albopictus (Skuse) Singh in vitro consisting of alterations in the nucleus and cytoplasmic degeneration (Belloncik and Gharbi-Said 1977).

Dresner (1950) and West and Briggs (1968) reported that B. bassiana produced toxins in vivo and in vitro. Based on culture filtrate injections, isolates of B. bassiana recently isolated from a host insect produced higher toxin titres than did isolates repeatedly subcultured on artificial media (West and Briggs 1968). Highly virulent strains of B. bassiana produce higher levels of insect toxins than do less virulent strains (Sikura and Bevzenko 1972).

Beauvericin, a cyclodepsipeptide insect mycotoxin, has been isolated from culture filtrates of B. bassiana and P. fumoso-roseus (Hamill et al. 1969). Administered per os the toxin was harmless to B. mori larvae but toxic by injection. Bassianolide, an ionophore cyclodepsipep-



tide, isolated from B. bassiana and Verticillium lecanii (Fries) Link culture filtrates was toxic to B. mori larvae per os as well as by injection (Kanaoka et al. 1978).

Two classes of toxic extracellular proteases in culture filtrates of B. bassiana have been described by Kucera and Samsinakova (1968); one class of high molecular weight proteases with an acidic optimum pH and the other class of low molecular weight proteases with a basic optimum pH. It was suggested that the proteases may be toxic directly by damaging the hemolymph or indirectly by producing a toxic byproduct in the insect.

Culture filtrates of B. bassiana, P. fumoso-roseus and P. farinosus contained toxins inducing paralysis in L. decemlineata (Wojeichowska 1973). The age of the cultures influences the time of onset of the symptoms.

Reiss (1973) reported that the growth rates of larvae of Tenebrio molitor L. were reduced when the insects were fed wheat bread on which Penicillium expansum Link, A. flavus, A. niger or Cladosporium herbarum (Fries) Link were growing. The entomophthoraceous fungi produce insect toxins in both stationary and shake cultures (Prasertphon 1968 and Prasertphon and Tanada 1969). In G. mellonella larvae receiving intrahemocoelomic injections of culture



filtrates of either Entomophthora coronata (Cost.) Kevorkian, Entomophthora apiculata Thaxter, Entomophthora virulenta Hall and Dunn or Entomophthora thaxteriana (Petch) Hall and Bell mycotoxin activity was indicated by darkening of the larvae. Entomophthora coronata and E. apiculata produced toxins. The two species produced toxins in any culture medium capable of supporting their growth. The toxin, on the basis of water solubility, heat-lability and precipitation by 10% trichloroacetic acid, was probably proteinaceous (Prasertphon and Tanada 1969).

Prasertphon and Tanada (1969) stated that the toxin was entirely extracellular and not a product of autolysis. Sublethal levels of culture filtrates injected into G. mellonella larvae prolonged larval life, affected metamorphosis, pupation and silk production. Metamorphosis was inhibited in B. mori, Carpocapsa pomonella L., Hyalophora cecropia L., Peridroma saucia Hübner, and Pseudelatia unipunctata Haworth. The toxin caused hemocyte aggregation, hemolymph coagulation, vacuolation of the epithelial cells of the silk glands and shrinkage of the nuclear contents (Prasertphon and Tanada 1969).

Paschke (1965) reported that Reticulitermes flavipes Kollar infected with E. coronata died without extensive mycelial development suggesting a possible mycotoxin was



## Materials and Methods

### I. Insects

A. Eastern hemlock looper. Fourth instar larvae of the eastern hemlock looper, Lambdina fiscellaria fiscellaria, were used in the study. Eggs, supplied by the Forest Pest Management Institute (Canadian Forestry Service, Sault Ste. Marie, Ontario), were hatched and the larvae reared on the diet of Grisdale (1975) until 10 days into the fourth instar.

B. Eastern spruce budworm. Unless otherwise stated only sixth instar larvae of the eastern spruce budworm, Choristoneura fumiferana, were used. Larvae, supplied in the second instar (Canadian Forestry Service, Sault Ste. Marie, Ontario), were reared on the diet of McMorran (1965) until they were 6 days into the sixth instar.

C. Tenebrio molitor. Larvae of Tenebrio molitor L. obtained locally were reared from the third instar to the sixth instar on a diet of ground flour and carrots.

D. Rearing conditions. For each species of insect twenty larvae were reared per nylon covered Petri dish (9cm diam) containing the diet. All insects were incu-



involved. Claydon (1978) reported similar results for E. virulenta in Calliphora erythrocephala Meigen. The mycotoxins of E. virulenta were identified as azoxybenzoid compounds (Claydon 1978).



bated at  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ,  $65\%\text{RH} \pm 5\%\text{RH}$  with a photoperiod of 16h light and 8h darkness.

## II. Hemograms of selected stages of *C. fumiferana*

A. Total and differential hemocyte counts. The larvae and pupae were sorted according to sex and stage of development. The stage of larval development was determined by measuring head capsule widths using the scheme of McGugan (1954). The stages used included the third, fourth, fifth and sixth larval instars, prepupal and early and late pupal stages.

Larvae were chilled at  $4^{\circ}\text{C}$  for 30min prior to bleeding. Hemolymph collected from cuts near the mid-dorsum of the larvae was diluted by the method of Stephens (1963) using chilled Grace's insect tissue culture medium (Grace 1962, 1966; Appendix I) as the diluent. Total hemocyte counts (THC) were done using a hemocytometer. The classification scheme of Price and Ratcliffe (1974) was used to identify the hemocytes. Differential hemocyte counts (DHC) were made by placing a drop of diluted hemolymph between a slide and an elevated coverslip. In accordance with Wittig (1966) a minimum of 10 larvae were used for THC and DHC. At least 100 hemocytes per larva were identified. Based on the efficient sample size formula of Sokal



and Rohlf (1969) the above sample sizes permitted detection of significant differences in THC and DHC at the 5% level.

B. Hemolymph volume. Hemolymph volumes for the larval stages of both sexes were determined using the amaranth dye techniques of Breugnon and LeBerre (1976).

Amaranth uptake by the Malpighian tubules and muscularis was determined by injecting 4 $\mu$ l of a 1% amaranth solution using a Hamilton microvolume syringe (CR-700-200, Hamilton Company, Reno, Nevada) into 4 groups of larvae of equal mass and containing 10 larvae per group. Larvae were incubated for 5, 7, 10 and 15min and the amaranth level was determined as described by Breugnon and LeBerre (1976). The log concentration of amaranth plotted against time allowed the value at time zero to be obtained by extrapolation. The percentage of amaranth removed after 5min by an insect was determined and the hemolymph volume corrected for the uptake of the dye. Regression equations for hemolymph volume and larval mass were developed for both sexes.

Because the larval masses used in the THC and DHC studies differed by only  $\pm 2\%$  and the hemolymph volumes



for a given stage and sex differed by  $\pm 5\%$ , the hemolymph volumes were averaged and used to calculate the absolute hemocyte counts (AHC).

### III. Collection of hemolymph and its fractions for interaction studies with protoplasts.

Prior to bleeding, sixth instar larvae were chilled at  $4^{\circ}\text{C}$  for 30min, swabbed with 70% ethanol and surface-dried with sterile, dry, cotton swabs. Hemolymph collected from cuts in the larval mid-dorsum was pooled in chilled, sterile, glass containers and rapidly diluted with the chilled modified Grace's medium. The medium was further modified by the omission of vitamins but still contained 2.7% V/V fetal calf serum and will be referred to as "MGM". Rapid dilution reduced hemocyte aggregation and melanization. Occasionally, hemolymph was used directly without dilution.

Serum and hemocyte fractions for the spruce budworm larvae were prepared by centrifuging whole hemolymph at  $250\times g$  ( $4^{\circ}\text{C}$ , 10min). The resulting serum was removed and used in studies pertaining to carbohydrate inhibition of hemocytes, analysis of protozoan lysins, humoral-protoplast interaction, media studies and comparisons of ninhydrin-positive compounds and serum protein analysis of selected



stages in larval development for both sexes. The hemocyte fractions were used in the analysis of the effects of sucrose on hemocyte interaction with spores of Absidia repens Hagem and in protozoan lysis studies.

Details of the use of hemolymph and the serum and hemocyte fractions are presented separately in sections IV and V.

#### IV. In vivo and in vitro hemolymph experiments.

##### A. Test particles in hemocyte adhesion studies.

1. Fungi. The fungi used included Entomophthora egressa isolate 458 (obtained from the eastern hemlock looper) and isolate 521 (obtained from the spruce budworm), A. repens and Rhizopus nigricans Ehrenb.. The latter two fungi were maintained on potato dextrose agar (Difco). The protoplast stage of E. egressa was maintained in MGM according to the procedure of Dunphy and Nolan (1977a). The walled stages of E. egressa were obtained by growing protoplasts in the medium of Dunphy and Nolan (1977b). The sporangiospores of A. repens were collected by flushing the agar surface of 4 week old cultures with MGM. Hyphae, teased from nonsporulating cultures of R. nigricans were suspended in MGM.



All the above test particles were centrifuge-washed 3 times in 15ml of fetal calf serum-free MGM (4000xg, 4°C, 5min).

With the exception of E. egressa isolate 521, all test particles were used with both the eastern hemlock looper and the eastern spruce budworm. The use of isolate 521 was confined to studies with T. molitor larvae and the eastern spruce budworm. Unless otherwise stated, reference to protoplasts will mean protoplasts of isolate 521 of E. egressa.

2. Bacteria. Escherichia coli K12 Migula and Bacillus cereus Frankland and Frankland were grown in trypticase soy broth (Difco) for 120h prior to centrifuge-washing (10,000xg, 4°C, 10min) in 15ml aliquots of serum-free MGM for each of 3 washings.

3. Sephadex. DEAE-Sephadex beads (A-50-120, Sigma) and CM-Sephadex beads (C-25, Pharmacia, Uppsala, Sweden) were suspended in serum-free MGM. All pH adjustments were made with either 4N HCl or 4N KOH. At pH 4.2, 6.2 and 7.2, the DEAE resins were positively charged; whereas the CM beads were negatively charged at the latter two pH values and neutral at the former value.



B. In vivo test particle studies. Cold-fixed eastern hemlock looper and spruce budworm larvae were anesthetized with CO<sub>2</sub>. The injection site, the base of the metathoracic legs, was swabbed with 70% ethanol and dried with sterile swabs. All larvae received 10 $\mu$ l injections.

Larvae of both species received  $1.1 \times 10^3 \pm 0.2 \times 10^3$  protoplast cells/ml of their respective pathotypes of MGM. The spruce budworm larvae also received the same level of protoplasts of isolate 458. Control larvae were injected with 10 $\mu$ l of MGM. Larvae were incubated (see section I. A, B. D) and examined for protoplast development at 24, 48 and 72h.

Both types of bacteria were injected into spruce budworm larvae as described above. Each larva received  $1.3 \times 10^8 \pm 0.1 \times 10^8$  bacterial cells. Control larvae received 10 $\mu$ l of MGM. The fate of the test organisms was determined after 24h.

In order to test the short-term effects of foreign particles on the hemograms of sixth instar female larvae either  $1.95 \times 10^8 \pm 0.2 \times 10^8$  cells of E. coli or  $2.0 \times 10^8 \pm 0.3 \times 10^8$  protoplasts of isolate 521 were injected into the larvae in 5 $\mu$ l quantities. Control larvae received 5 $\mu$ l of MGM. Larvae were examined at 0, 5, 10, 20, 30



and 60min for THC and test particle levels. The THC values were compared to those of larvae that were not injected. To determine the feasibility of test particle growth in vivo, control MGM suspensions containing the number of test particles injected into the larvae were sampled at the above times and the cell levels determined. For each treatment regime 20 larvae were sampled at the designated incubation times.

Because of the problem of injecting large numbers of larvae, the remaining experiments were conducted in vitro.

C. In vitro test particle studies.

1. Labdina fiscellaria fiscellaria.

(i) Monolayer technique. The ratio of test particles (except for hyphal stages) to hemocytes was 1:100 in the studies described in this and the following section. The monolayer technique (Ratcliffe 1975) used suspensions of test particles in known volumes of MGM to dilute hemolymph. This reduced the chances of culture artifacts, due to prolonged in vitro manipulations, influencing the results. This procedure was used only for E. coli test particles.



To gain insight into the activity of the hemocytes against hyphae of R. nigricans, a modification of the monolayer technique was used in which nondilute hemolymph was run under an elevated coverslip covering hyphae mounted in a minimum volume of MGM. During incubation at 12°C on a cold stage mounted on a microscope, the hemocytes were observed continually for the first 30min and then after 24h.

(ii) Microcapacity-centrifuge tube method.

This technique was used for all test particles and consisted of adding 2µl of test particle suspension to 2µl of hemolymph in 246µl of serum-free MGM in 400µl microcapacity centrifuge tubes (MccT) (Canlab, Toronto, Ontario). Control suspensions consisted of hemocyte suspensions (246µl) with 2µl of test particle-free and serum-free MGM and test particles in such a medium without hemolymph. Nylon fibers of equal length (100µm) were added to several samples containing protoplasts and hemocytes as well as the corresponding controls. All suspensions were incubated in darkness at 25°C for 30min on a horizontal shaker (100rpm).

2. Choristoneura fumiferana.

(i) Microcapacity-centrifuge tube technique.



The Mcct technique was used in all subsequent experiments. The following ratios were used; hemocytes:resin beads, 100:1; hemocytes:protoplasts, 20:1; hemocytes:hyphal bodies, 10:1; hemocytes:sporangiospores of A. repens and R. nigricans, 70:1 and for the carbohydrate inhibition studies, hemocytes:spores of A. repens, 90:1.

(ii) Protoplast inhibition of hemocytes.

To determine if the protoplast stage of E. egressa isolates inhibited hemocyte adhesion to the hyphal stages of E. egressa by secretion of inhibitors, MGM washed mycelia of both isolates were separately fragmented in a Waring blender in either MGM in which no previous protoplast growth had occurred or in MGM in which either isolate had grown for 72h. A total of six different regimes were established. The absorption of each sample at 500nm was adjusted to  $0.270 \pm 0.002$  using the corresponding MGM type. Aliquots (100 $\mu$ l) of hyphal suspensions were added to 150 $\mu$ l of hemolymph. The final suspensions were incubated using the Mcct technique for 30min. The number of freely-circulating hemocytes was determined prior to examination of the hyphal fragments.

(iii) Hemolymph inhibition of hemocytes.

Protoplast cells may avoid hemocyte attachment by being coated with host hemolymph constituents, thus, masking their



foreign nature. Using the procedure and levels of hemocytes, protoplasts and inoculum volumes of section IV. C. 1. ii. both hemocytes and protoplasts were centrifuge-washed in either sucrose or trehalose (both at 350mOsM/Kg, pH6.2) and incubated together in the corresponding solutions. The number of granular cells per protoplast and the proportion of protoplasts with attached granular cells were determined.

(iv) Hemocyte-test particle interaction with phenylthiourea. Hemolymph with phenylthiourea (a known inhibitor of hemocyte activity) (PTU, 4µg/ml) and without PTU was used to analyse the adhesion of spores of A. repens and R. nigricans to the granular cells in an attempt to detect specificity in spore-hemocyte adhesion in inhibited and noninhibited hemocyte test particle adhesion. The procedure in section IV. C. 1. ii. was used in this and the following two experiments.

(v) Test for hemolymph opsonins. Hemocytes, centrifuge-washed in either MGM or larval serum, were re-suspended in 290µl of corresponding MGM or larval serum. To this 10µl of spores of A. repens washed in either MGM or larval serum were added to the corresponding hemocyte suspensions. The final hemocyte:spore ratio was 90:1. The number of spores per granular cell and the percentage of granular cells with spores were determined.



(vi) Carbohydrate inhibition of hemocyte activity. To assist in identifying the possible nature of the receptors on the granular cells, the effects of carbohydrates on the adhesion of A. repens spores to hemocytes was studied using spores that were centrifuge-washed (4000xg, 4°C, 10min) with solutions of the following carbohydrates; trehalose, sucrose, D-fucose, D-fructose, D-arabinose, D-mannose, D-galactose, D-glucose, D-glucosamine and N-acetylglucosamine. The final osmolality of all test solutions was 350mOsm/Kg. When necessary the pH was adjusted to 6.2 using 0.01N HCl. The spore suspensions (290µl) were added to 10µl of freshly collected hemolymph. Controls consisted of hemolymph (10µl) diluted with 290µl of spruce budworm serum. This was a valid control system based on the results of section IV. C. 2. v. The number of spores/granular cell, granular cells/aggregate, hemocyte aggregation frequency and total available granular cell surface area were determined for each sample after 30min incubation. Ten replicates using hemocytes from different individual larvae were used for each carbohydrate.

D. Observation of hemocytes.

After incubation the suspensions were sampled and the hemocytes were allowed to attach to coverslips



for 5-10min, on a 7-10°C cold stage on a microscope, to form a monolayer. In work with bacteria and fungal spores, the coverslips were rinsed with serum-free MGM to remove most of the unattached test particles. In the case of fungal hyphae suspensions, the hyphae were gently agitated in serum-free MGM to remove hemocyte debris. The protoplasts, hyphal body suspensions and nylon fiber suspensions were diluted 1:100 with the tissue culture medium to reduce cluttering of the field by non-adhering hemocytes. The hemocytes and test particles were regarded as attached if pressure applied to the coverslip failed to dislodge the hemocytes or test particles.

All of the above test cell-hemocyte monolayers produced by either the MccT technique or the direct monolayer technique were photographed using bright-field or phase contrast microscopy. Those photomicrographs of MccT samples were labelled "MccT" in the figure legends and those of the direct monolayer samples were labelled "ML" in the figure legends. Monolayers prepared after in vitro incubation of the test particles were designated "in vitro" in the legends.

## V. Protozoan lysins

### A. Preparation of the protozoa. The protozoa



Paramecium caudatum Ehrenberg and Euglena gracilis Klebs were selected on the basis of foreignness to the spruce budworm and availability to assess how the spruce budworm hemolymph responded to mobile, foreign particles. Both protozoa (Boreal Laboratories, Mississauga, Ontario) were grown in hay infusions. The organisms were centrifuge-washed 3 times (100xg, 4°C, 5min) in MGM. The final concentration of protozoa prior to mixing with the hemolymph samples was  $2.0 \times 10^4 \pm 0.2 \times 10^4$  cells/ml. Centrifugation was not harmful to cells of P. caudatum as judged by the resumption of normal ciliary activity within seconds after centrifugation and an increase in protozoan levels up to  $5.7 \times 10^4 \pm 1.2 \times 10^4$  cells/ml after 36h of incubation in MGM. Mobility was restored to E. gracilis within seconds of centrifugation.

B. Hemolymph with and without phenylthiourea.

Hemolymph (100ul) with phenylthiourea (PTU, 4ug/ml) and without PTU was added directly to pelleted cells of either P. caudatum or E. gracilis. The suspensions were incubated on a hemocytometer at 25°C and 7-10°C on a cold stage microscope. The effects of the hemolymph were assessed using both organisms. However, it was only for samples with P. caudatum that the percentage of stationary cells was determined in MGM control samples and in hemolymph plus MGM, all containing PTU.



C. Hemolymph dilution regimes. A hemolymph:MGM dilution regime of 6:5, 7:5 and 8:5 (250 $\mu$ l final volume) was incubated as described in section IV. C. 1. ii. Only P. caudatum was used as the test organism in this and the following experiments pertaining to protozoan lysins using the procedure of section IV. C. 1. ii.

D. Heating and dialyzing larval serum. Several serum samples were heated to 60°C for either 5 or 30 min. Other samples (150 $\mu$ l) were dialysed against 2l of 10mM 2- (N-morpholino) ethanesulfonic acid (MES) (pH6.2) for 72h, freeze-dried and reconstituted to the original serum volume with 345mOsm/Kg sucrose (pH6.2). The final osmolality and pH were 350mOsm/Kg and 6.2 respectively. These values represented the osmolality and pH of female spruce budworm larval hemolymph at 25°C as reported later in the text.

E. Hemocyte preparation. Hemocyte pellets were centrifuge-washed 3 times (250xg, 4°C, 1min) in MGM. The exclusion of trypan blue by 98% of the hemocytes indicated that the cells survived this treatment. The hemocyte concentration was adjusted to  $3.0 \times 10^6 \pm 0.2 \times 10^6$  cells/ml of MGM prior to mixing with the protozoan suspensions.



The serum and hemocyte experiments were repeated using protozoan suspensions containing PTU (4 $\mu$ g/ml).

VI. Comparative physiology of two isolates of *Entomophthora egressa*

A. Inoculum preparation. Except for the growth studies using carbon dioxide (CO<sub>2</sub>), inocula were prepared as described by Dunphy and Nolan (1979). For the latter studies 15ml of MGM was placed in 30ml capacity tissue-culture flasks (Falcon Division, Becton-Dickinson, Oxnard, California).

The concentration of protoplasts in the inoculum varied with the experiment but was the same for both isolates. The protoplast level for growth studies in MGM in shaken cultures in air (Dunphy 1977) was  $1.4 \times 10^4 \pm 0.1 \times 10^4$  cells/ml; in the CO<sub>2</sub> experiments the level was  $1.4 \times 10^5 \pm 0.2 \times 10^5$  cells/ml and for medium A, B, and C (defined in section VI. B.) the level was  $1.8 \times 10^3 \pm 0.3 \times 10^3$  cells/ml.

B. Media. Coagulated egg-yolk was prepared by the method of Muller-Kogler (1959). Other media included MGM, the simplified growth medium of Dunphy and Nolan (1977b) but lacking vitamins (medium A), and the following



additional modifications; decreased level of  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  from 1000mg/l to 750mg/l (medium B), increased KCl from 2240mg/l to 4100mg/l (medium B<sub>1</sub>), a composite medium reflecting both changes in  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  and KCl (medium B<sub>2</sub>), medium B<sub>2</sub> with a decrease from 700 to 400mg/l of glucose (medium C), medium B<sub>2</sub> with an increase from 400 to 700mg/l of fructose (medium C<sub>1</sub>) and a composite of C and C<sub>1</sub> (medium C<sub>2</sub>) (consult Appendix II for further clarification).

Because the protoplast growth yields in the media of the B series were statistically equivalent the results were pooled and considered as those of medium B. Similar equality was observed for the C series of media and the results were reported as those of medium C.

All the media contained heat-inactivated fetal calf serum (FCS, Grand Island Biological Company, Burlington, Ontario). The osmotic pressure was kept constant by the addition or deletion of varying amounts of sucrose (Dunphy and Nolan 1977b).

The generation of CO<sub>2</sub> gas was achieved by using the BBL Anaerobic Gas Pak system (Becton, Dickinson and Company, Cockeysville, Md.). The CO<sub>2</sub> levels were atmospheric (0.03%), 3%, 6% and 10%. No change in the internal pressure of the gas packs was detected under



these conditions. The pH of MGM remained at 6.2.

C. Cell yields and chemical analysis. All growth curves and morphological states were determined as reported by Dunphy and Nolan (1977a, 1979). The fungal cells were removed from only medium A by centrifugation (5,000xg, 4°C, 5min). The supernatant was analysed for changes in pH, osmolality, total protein by the method of Lowry et al. (1951), free ninhydrin-positive compounds (NPC) and glucose uptake (Dunphy 1977).

To determine the NPC content of the protoplast stage after 48h in MGM the cells were centrifuge-washed 3 times in a sucrose solution (360mOsm/Kg, pH6.2). The protoplasts were lysed by ultrasonication for 3-30sec intervals while chilled at 4°C. Cellular debris was removed by centrifugation (10,000xg, 4°C, 15min). Based on the volume of lysate and the number of protoplast cells involved the level of soluble NPC were determined in picomoles/1000 protoplast cells.

The 48 h / protoplast stage of both isolates of E. egressa in MGM and the 120 hold fusion sphere stage of isolate 458 and the spherical hyphal body stage of isolate 521 were centrifuge-washed and lysed as above. The lysate was analysed for total protein content.



The dry mass of the samples was determined by the method of Nolan (1976). Protein content was expressed in mg/g of dry fungal mass.

Five biological samples were used for each point on the growth curves in this and the following sections. Seven separate biological samples were used for analysis of the components of medium MGM and selected stages of fungal development.

VII. Analysis of granular cell and protoplast cytoplasmic membranes.

The granular cells of the spruce budworm larvae did not adhere to the protoplasts of either I458 or I521 of E. egressa. Previous experiments implied that this was due to the surface of the protoplasts. Treatment of the protoplasts with proteolytic enzymes could indicate if surface proteins on the protoplasts were preventing the attachment of granular cells.

A. Trypsin.

1. Trypsin assays. Trypsin (Sigma Chemical Company, St. Louis, Mo.) was assayed as described by Rick (1974) using a Tris-HCl buffer (pH7.5) as the solvent. Because



of the osmotic frailty of the protoplasts, the enzyme was assayed using a sucrose-Tris-HCl buffer (pH7.5). The activity of the enzyme was also determined after filter sterilizing the trypsin-sucrose-Tris-HCl buffer. This buffer system was used in all trypsin assays.

## 2. Protoplasts of *Entomophthora egressa*.

(i) Trypsinized protoplast cells. Protoplasts of *E. egressa*, isolate 521, previously centrifuge-washed 3 times in sucrose were exposed to one trypsin unit in 2ml of the sucrose-buffer used in section VII. A. 1. A trypsin unit is the amount of enzyme hydrolysing hemoglobin releasing 1 m mole of tyrosine per minute (Rick 1974). After incubating for 0, 10, 20, 30 and 40min, 500ul samples were aseptically centrifuge-washed 3 times (150xg, 4°C, 2min) in MGM. The MGM was found to inactivate trypsin. The final suspension was analysed for protoplast concentration and the percentage of cells in spindle form. Using the inoculum containing  $5.1 \times 10^5 \pm 0.1 \times 10^5$  cells (in 0.1ml) growth curves were determined for each incubation period. The supernatant of the first centrifugation was analysed for tyrosine level as described by Rick (1974) to ensure trypsin activity. All of these data were used to determine the optimum exposure time of the protoplasts to trypsin.



(ii) Hemocyte interaction with trypsinized protoplasts. Protoplasts exposed to trypsin for 10 and 20min were centrifuge-washed (see section IX. A. 2. i.) and exposed to either hemolymph or MGM washed hemocytes using the McCT technique. Hemocytes and protoplasts were examined and compared to the results obtained with non-trypsinized protoplasts and hemolymph and hemocytes.

### 3. Hemocytes.

(i) Trypsinized hemocyte viability. Hemocytes washed three times in a sucrose-MES solution were exposed to 2ml of trypsin solution (1 unit/2ml) for 10 and 20min at 25°C. The final hemocyte concentration, when resuspended in the trypsin solution, was  $1.2 \times 10^4 \pm 0.2 \times 10^4$  cells/ml. After exposure to the enzyme, the hemocytes were centrifuge-washed in MGM to inactivate and remove trypsin. Both trypsinized groups of hemocytes excluded trypan blue from 95% of the hemocytes and 98% of the granular cells. In the control samples 98% of total hemocytes and 94% of the granular cells excluded trypan blue.



(ii) Trypsinized hemocytes and spores of *Absidia repens*. As described in section IV. C. 2. the 2 groups of trypsinized hemocytes and non-trypsinized hemocytes were incubated with spores of *A. repens*. The hemocyte:spore ratio was 1100:62. The number of spores per granular cell and the number of granular cells with spores were determined.

B. Papain.

1. Papain assays. Papain (Sigma Chemical Company, St. Louis, Mo.) was assayed as described by Arnon (1970) using a Tris-HCl buffer (pH8.0). Because of the nature of the protoplasts mentioned in section IX. A. 1., the assay was redone using a sucrose-Tris-HCl buffer. Sucrose, like the protein hydrolysates on which the assay was based, absorbed light at 280nm. To facilitate the comparison of the enzyme activity between papain in Tris-HCl buffer and in sucrose-Tris-HCl buffer both types of enzyme preparations were chromatographed on a Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (4cm x 1.5cm, Tris-HCl buffer, pH8.0; elution rate = 0.4ml/min). The small pore size of the column resin effectively retained the sucrose. The enzyme activities of both systems were compared by analysing the similarities of the elution profiles measured at 280nm absorption



and the magnitude of the large fractions 7 and 14. This assay procedure was repeated for filter sterilized papain to ensure enzyme activity in the filtrate.

## 2. Protoplasts of *Entomophthora egressa*.

(i) Protoplast viability. Pelleted protoplasts of isolate 458 and isolate 521 were aseptically exposed to either 1 unit of sterile papain (1 unit is the activity which increases 1 unit of absorbancy at 280nm per min of digestion) for 10min as described in section VII. 2. i. or to sterile solutions of either papain activators or a solution of activators plus 5ml of 10% trichloroacetic acid (TCA). The latter two treatments were to assess possible harm to the protoplasts due to the papain activators (cysteine and ethylenediaminetetraacetic acid) and/or the inactivation of papain with TCA. In the former suspension the activated papain was inactivated by 5ml of TCA. The protoplasts of both isolates were aseptically centrifuge-washed as described in section VII. A. 2. i.. Viability was assessed using the results of growth experiments.

(ii) Hemocytes exposed to papain treated protoplasts. Following the procedure of section IX. 2. ii. protoplasts of both isolates exposed to papain as described above were exposed to either hemolymph or sucrose washed



hemocytes. Using the McCT procedure the results were compared to the protoplast controls.

VIII. Comparison of serum proteins of selected stages of male and female spruce budworm.

The free NPC of the serum of the third, fourth, fifth and sixth female larval instars and the fifth and sixth male larval instars at ages corresponding to those of section II. A. were determined according to Dunphy et al. (1977). Osmolalities of the sera of the fifth and sixth instars of both sexes were determined using an osmometer (Advanced Osmometer, Model 31, Needham Heights, Mass.).

The procedure of Lowry et al. (1951) was used to determine the total serum protein content of the larval and pupal stages used in section II. A. in addition to the early sixth instar stage which was 25 days past the third instar.

Serum samples (5 $\mu$ l) were electrophoresed according to the method of Cheung et al. (1978). With the exception of designated frozen-serum samples from sixth instar female larvae (Parson, 1978), only fresh serum was used. Generally, 3 serum samples representing pooled hemolymph from 50 larvae were used in these serum analyses. Eight



serum samples were used for work on the sixth instar female larvae.

IX. Protoplast interaction with humoral and cellular hemolymph fractions.

A. Exposure of hemolymph and serum to protoplasts.

1. Living protoplasts. Unless otherwise stated protoplasts refer to those of isolate 521 of E. egressa.

Hemolymph (20 $\mu$ l) from female fifth and sixth larval instars was added to either 40 $\mu$ l of the sucrose-MES solution of Dunphy and Nolan (1979) in microcapacity centrifuge tubes or 20 $\mu$ l of hemolymph to 40 $\mu$ l of the sucrose-MES solution containing  $5.1 \times 10^3 \pm 0.1 \times 10^3$  protoplasts/ml in MccT. The protoplasts had been previously centrifuge-washed (5000xg, 4°C, 5min). The protoplast: hemocyte ratio was 1:1000. The protoplast controls consisted of 40 $\mu$ l of protoplast suspension added to 20 $\mu$ l of the sucrose-MES solution. The hemolymph control consisted of added 20 $\mu$ l of hemolymph to 40 $\mu$ l of the sucrose-MES solution.

Using the MccT technique (section IV. B. 1. ii) and after 2h of incubation, the suspensions were centrifuged



(5000xg, 4°C, 15min) and the supernatant analysed for total protein and electrophoresed using the procedures of Lowry et al. (1951) and Cheung et al. (1978), respectively.

The experiment was repeated using only sixth instar female serum and the hemocyte fractions, separately, to determine the possibility of humoral-cellular interaction with the protoplasts.

2. Killing of protoplasts by cold-fixing and ethanol treatment. To determine if the results of section IX. A. 1. were due to the hemolymph or serum and not some aspect of metabolism, it was necessary to repeat the experiments using intact but non-viable protoplasts.

Two methods were used to kill the cells:

- (1) incubation of the protoplasts at 4°C for 18h in a sucrose-MES buffer and,
- (2) chilling the cells for 18h as in method "1" except that the buffer contained 4% (V/V) ethanol.

The protoplasts were centrifuge-washed 3 times in MGM. Protoplast viability was assessed by adding 0.1ml of protoplast suspension ( $1.1 \times 10^3 \pm 0.1 \times 10^3$  cells/ml) to 5-10ml volumes of MGM in tissue culture flasks. Controls



consisted of protoplasts incubated at 20°C for 18h in MGM followed by centrifugation and re-inoculation of equal protoplast levels into MGM. After 24h of stationary incubation, protoplast concentrations were determined.

To determine if the treatments might induce hemocyte adhesion to the protoplasts, 20 $\mu$ l of hemolymph was added to protoplasts from each treatment.

The experiments of section IX. A. 1. were repeated using ethanol-killed protoplasts and whole hemolymph.

3. Whole protoplast cells, lysed protoplasts and heated protoplast lysate. Killed protoplasts influenced the electrophoretic profiles in a manner identical to the living protoplasts in addition to lowering the total protein levels. The possibility existed that an active surface membrane protease might account for the effects.

Whole protoplast cells, both living and dead protoplasts, lysed protoplasts (see section VI.C.) and protoplast lysate heated to 80°C for 30min obtained from a common stock culture ( $4.0 \times 10^5 \pm 0.1 \times 10^5$  cells/ml), were added as 40 $\mu$ l portions to 20 $\mu$ l of larval serum (from sixth instar female larvae) in 5ml Pyrex glass centrifuge tubes.



The tubes were shaken at 50rpm on a Dubnoff metabolic shaking incubator (Precision Scientific, Ontario) at 25°C for 0, 1, 2 and 3h. Samples were centrifuged to remove debris and the total supernatant protein determined by the method of Lowry et al. (1951).

B. Exposure of protoplasts to dilute sera.

1. Serum dilution regimes. To study the effects of short-term interaction of serum and protoplasts, female sixth instar serum containing PTU (4µg/ml) was diluted to 50% and 3% of its former concentration using MGM without PTU. Long-term studies used male and female sixth instar larva serum with PTU diluted to 1%, 2% and 3% of its initial concentration. Control samples consisted of MGM plus PTU (4µg/ml) diluted to the levels of the sera using MGM lacking PTU. All solutions were filter-sterilized using Millex microfilters (Millipore, 0.22µm).

2. Short-term and long-term exposure studies.

Using the MccT technique aseptically (section IV. C. 1. ii) 300µl of 16h protoplasts ( $6.5 \times 10^4 \pm 0.2 \times 10^4$  cells) were added to 700µl of the two serum based and MGM control media. Samples (150µl) were aseptically centrifuge-washed 3 times in 500µl of MGM after 0.5h and 1h of incubation. The final 500µl suspensions were added to 9.5ml



of MGM in tissue culture flasks. The cells were incubated in darkness as stationary cultures ( $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) during analysis of growth rates.

Long-term exposure consisted of adding 200 $\mu\text{l}$  of 16h protoplasts ( $5.5 \times 10^4 \pm 0.8 \times 10^4$  cells) to 9.8ml of MGM containing 2% larval serum in tissue culture flasks. The cells were incubated for growth-curve analysis as described in the short-term exposure studies. The number of protoplast cells per chain was also determined.

For both the short-term and long-term studies, four replicates were used for each data point.

#### X. Larval serum simulation.

Because of differences between isolates 458 and 521 in various media (section VI) it was proposed that the two isolates may not be equally adapted to the host hemocoel. By devising media reflecting male and female larval serum, an attempt was made to determine if a given isolate was equally adapted to the serum of both sexes.

A. Simulation of male and female larval serum. The NPCs of male and female sixth instar larval serum replaced the NPCs of MGM as described by Dunphy et al. (1977). The



osmolalities of the media were adjusted using sucrose to reflect the values corresponding to the serum of the male and female larvae. Using the incubation conditions of section VI. C., 0.1ml of protoplasts of isolate 521 containing  $2.2 \times 10^4 \pm 0.1 \times 10^4$  protoplasts/ml was added to 49.9ml of either "male" or "female" based media. After 72h incubation, when growth levels reached  $2.5 \times 10^4 \pm 0.2 \times 10^4$  protoplasts/ml, 0.1ml from each type of medium was transferred to 49.9ml of the corresponding medium and was incubated as described in section VI. C.. The growth rates were compared to the rates in MGM.

B. Lobster serum.

1. Preparation of lobster serum. Serum of Homarus americanus (L.) (Grand Island Biological Company, Burlington, Ontario) was believed to be chemically closer to the insect serum than to fetal calf serum (FCS). Six ml of lobster serum and FCS were dialysed against 13L of 10mM MES-buffer (pH6.2) for 72h. The dialysates were freeze-dried, redissolved in 310mM sucrose and electrophoresed as described in section VI. C..

2. Media. The lobster serum and FCS dialysates were reconstituted such that by the addition of equal volumes of the solutions to the media based on both sexes



resulted in equal levels of total protein for all types of media.

The media were inoculated and incubated as described in section XI. A.. The number of protoplasts per chain and the protoplast growth patterns and rates were analysed.

3. Lobster serum experiment. Tightly packed 5ml volumes of sucrose-washed protoplasts were added to 10ml of reconstituted lobster serum. The suspensions were incubated at 10°C for 30min. The serum, separated from the protoplasts by centrifugation (10,000xg, 4°C, 10min), was added to freshly prepared protoplasts. This procedure was repeated 5 times. The absorbed serum was added to the "male" and "female" based media as previously described in section B. 2.. The effects of these media on the growth rates of the protoplasts were determined.

## XI. Toxin studies.

### A. Initial toxin studies.

#### 1. Injection of spent medium components.

Injection of 6µl of MGM containing 48h protoplasts of isolate 521 and isolate 458 induced rapid paralysis in



male and female sixth instar spruce budworm larvae.

To determine the source of the toxin, the protoplasts of isolate 521 were removed from the medium by centrifugation. The cells were centrifuge-washed 5 times in MGM. The initial spent medium was filter sterilized to ensure the removal of particulate matter greater than  $0.2\mu\text{m}$  diam. Six  $\mu\text{l}$  of this medium and  $3\mu\text{l}$  of the medium diluted  $1/10000$  with MGM were injected into female sixth instar larvae. Control larvae received  $6\mu\text{l}$  and  $3\mu\text{l}$  of MGM. The washed protoplast suspensions containing  $2.7 \times 10^6 \pm 0.1 \times 10^6$  protoplasts/ml of MGM were injected into larvae in  $6\mu\text{l}$  volumes. The insects were incubated in Petri dishes ( $2.5\text{cm}$  diam) containing moistened sterile filter paper. (Refer to section I. for incubation conditions) The percentage of larvae exhibiting total paralysis, partial paralysis, total movement and bacterial development were determined over a 102h incubation period.

2. Comparison of protoplast isolates. Spent culture media resulting from the 48h growth of protoplasts of isolate 521 produced from mycelia and conidia were compared to their counterparts from cultures of isolate 458. The level of protoplasts in all cultures used as inoculum was  $2.1 \times 10^4 \pm 0.1 \times 10^4$  protoplasts/ml. Only  $3\mu\text{l}$  of each medium diluted  $10^{-4}$  was tested. The percentages of larvae



with total paralysis was determined for each treatment.

Washed protoplasts from the selected fungal sources at levels of  $2.7 \times 10^6 \pm 0.2 \times 10^6$  protoplasts/ml of MGM were injected into larvae in 6 $\mu$ l volumes. The insects were observed for signs of mycosis and the production of in vivo toxins.

3. Centrifugation effects on the protoplast isolates. Because protoplast-injected larvae failed to develop any mycoses, it was believed that the protoplasts may have been damaged or altered during centrifuge-washing. All isolates of E. egressa tested produced toxins as judged by the occurrence of paralysis. A centrifugation procedure involving less force could reduce protoplast damage and facilitate the detection of toxins produced in vivo. The centrifugation regime tested was 10xg, 25xg, 65xg, 120xg and 255xg for either 5 or 7min. The assessment of the effects of these conditions on each isolate was based on the percentage of spindle cells after 5min incubation in stationary MGM and the growth rates. The inoculum levels of the centrifuged cells and their corresponding controls were  $1.4 \times 10^6 \pm 0.1 \times 10^6$  cells/ml.

Protoplasts of isolate 521 from conidia of E. egressa centrifuged at 65xg for 7min were chosen to repeat the



protoplast injection study since this isolate best tolerated treatment.

B. Differential hemocyte counts. Larvae receiving 3ul of the spent MGM diluted  $10^{-4}$  were bled 18h post injection for DHC analysis. Twenty-three larvae were used for each sample.

C. Toxin production. Cultures of 18h protoplasts of isolate 521 of conidial origin ( $0.1\text{ml}$ ,  $1.2 \times 10^4 \pm 0.1 \times 10^4$  cells/ml) were added to  $9.9\text{ml}$  of MGM in tissue culture flasks and incubated in stationary culture at  $20^\circ\text{C}$  for 138.5h. Samples of media were analysed for toxicity, pH, osmolality and protoplast concentration. Five replicates were used for each individual determination.

D. Osmolality analysis. There was no correlation between changes in medium osmolality and insect paralysis. To determine the possible influence of osmolality on larval paralysis, 3ul of either sucrose or trehalose solutions (pH6.2 at 300, 350, 570 and  $800\text{mOsm/Kg}$ ) were injected into sixth instar larvae. Control larvae received 3ul of MGM.

E. Nature of the toxin. Media containing the toxin were heated to  $65^\circ\text{C}$  and  $75^\circ\text{C}$  for 15 and 60min. The pH and osmolality were checked after the media had cooled to  $25^\circ\text{C}$ .



The level of paralysis in larvae receiving 3 $\mu$ l of such media was determined 2h post injection. Ten ml of 10% TCA was added to 10ml of toxic medium and 10ml of MGM. The precipitates were removed by centrifugation and the pH of the supernatant adjusted to 6.2 using 4N NaOH. The levels of larval paralysis were compared.

### XII. Statistical analysis.

The major statistical procedure used throughout the study was the Student-t-test. Other statistical procedures consisted of analyses of variance, Student-Newman Keuls test of significance and the efficient sample size formula of Sokal and Rohlf (1969). The procedure used will be designated in the appropriate section of the thesis.

All tabular and graphic values indicate the mean and standard error of the mean.



## Results and Discussion

### I. Hemograms of selected stages of *C. fumiferana*

Prior to an analysis of parasite-host interaction it was necessary to determine the hemograms of the host insects. The hemocyte profiles for *L. fiscellaria fiscellaria* were determined by Boiteau and Perron (1977). There were no such analyses for *C. fumiferana*.

#### A. Results.

1. Hemocyte descriptions. Five types of hemocytes were identified in the insect developmental stages examined: plasmatocytes, granular cells, spherule cells, prohemocytes and oenocytoids. Intermediate forms, classified as other, were also detected.

The granular cells (Plate 1, Fig. 1), even though adhering to glass surfaces by means of protoplasmic extensions (Plate 1, Fig. 1, arrows), still retained an overall circular to oval shape. These small cells (Table I) had centrally located nuclei, although subcentric nuclei were occasionally detected. The granular cells readily adhered to neighboring granular cells unless the hemolymph was rapidly diluted.



## Plate 1

- Fig. 1. Granular cell with protoplasmic extensions (arrows). Phase contrast. X 650.
- Fig. 2. Plasmatocyte with ruffled membrane (arrow) and central nucleus. Phase contrast. X 800.
- Fig. 3. Plasmatocyte with multiple ruffled edges. Phase contrast. X 900.
- Fig. 4. Granular hemocyte (single arrow), plasmatocyte (double arrow), prohemocyte (triple arrow). Nomarski. X 650.



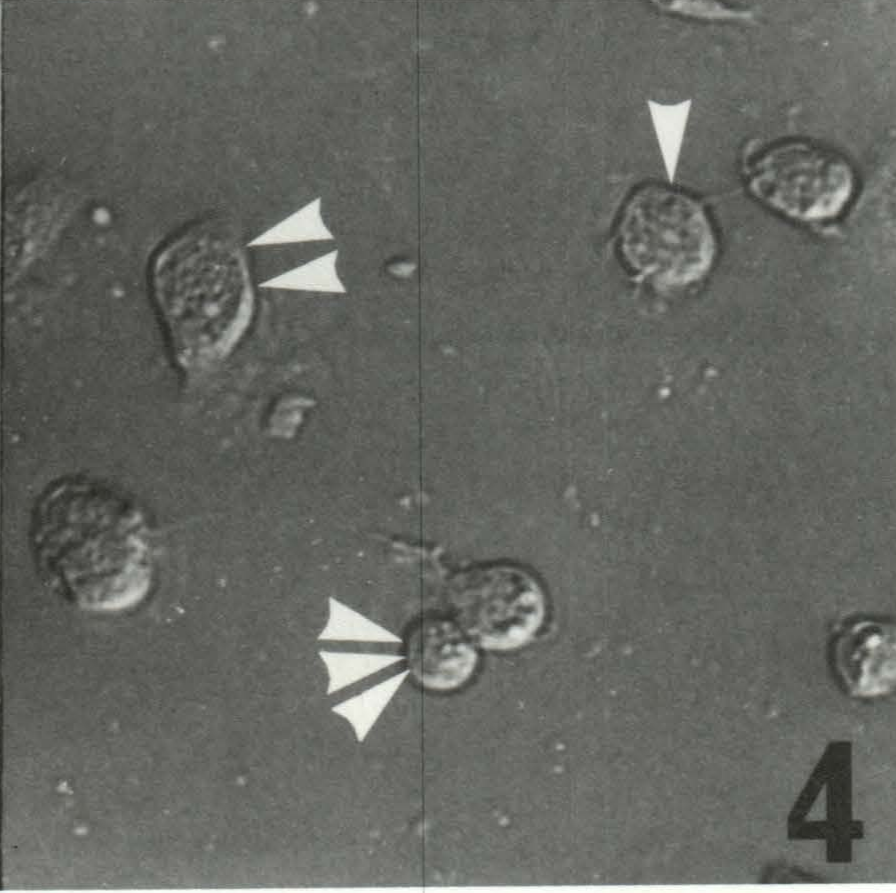
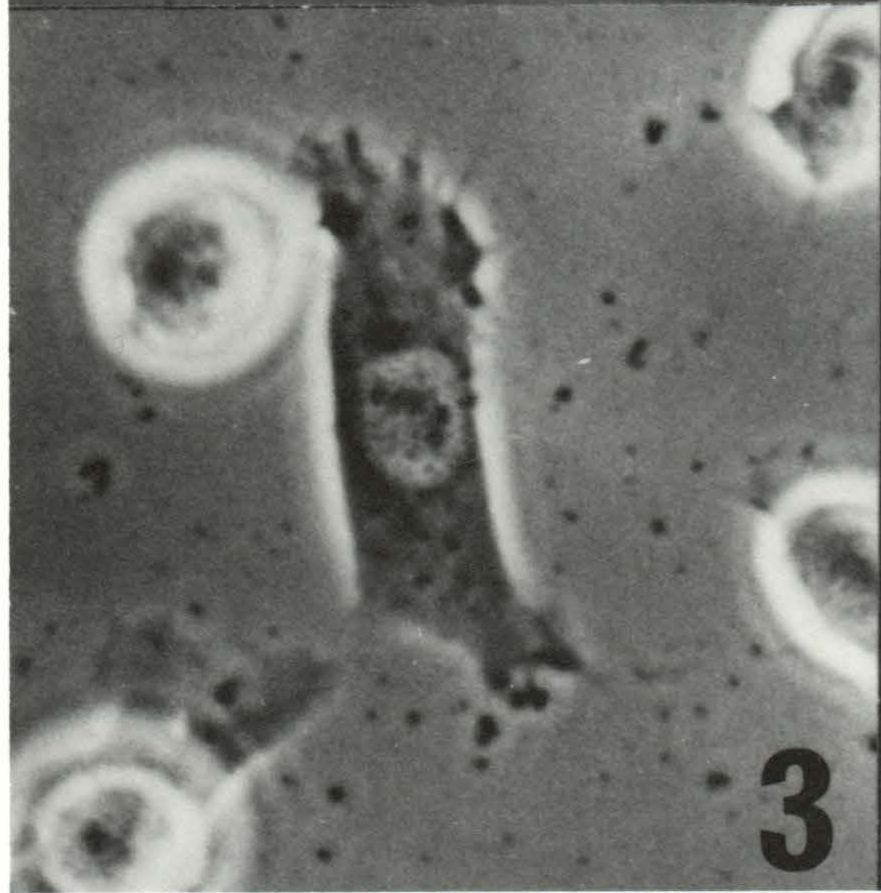
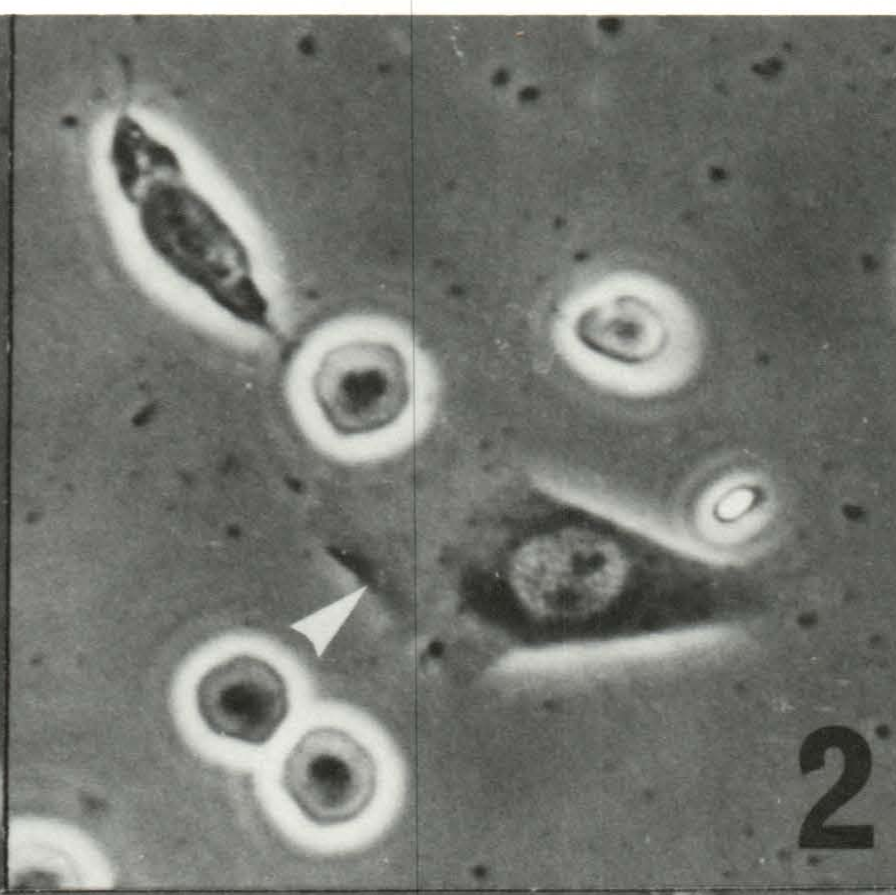
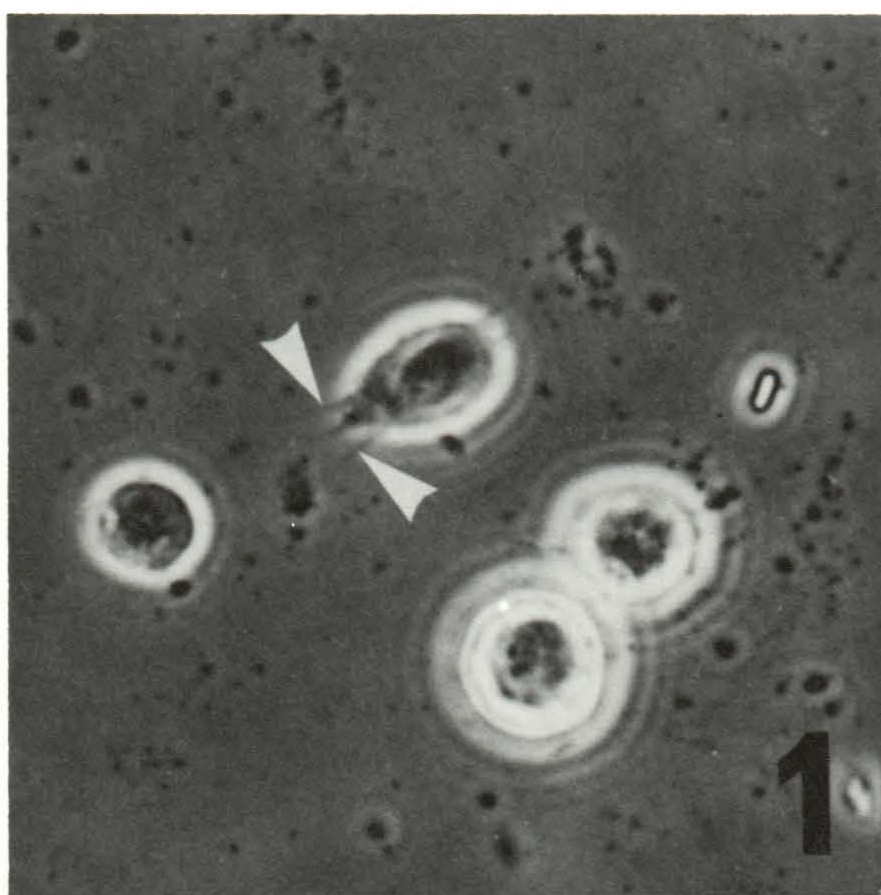




Table I  
Dimensions of hemocyte types  
from Choristoneura fumiferana<sup>a</sup>

Dimension	Type of hemocyte				
	Prohemocyte	Plasmatocyte	Granular cell	Spherule cell	Oenocytoid
Length	9.6 $\pm$ 0.1	48.2 $\pm$ 0.9	10.9 $\pm$ 1.3	16.1 $\pm$ 0.6	28.4 $\pm$ 0.1
Width	9.6 $\pm$ 0.1	23.9 $\pm$ 0.8	10.0 $\pm$ 1.0	7.3 $\pm$ 0.1	17.5 $\pm$ 0.1
Diameter of nucleus	3.9 $\pm$ 0.4	12.0 $\pm$ 0.1	4.3 $\pm$ 0.3	— <sup>b</sup>	9.1 $\pm$ 0.0

<sup>a</sup>All dimensions in  $\mu\text{m}$ , values represent the mean  $\pm$  standard error of the mean for 10 larvae and 100 hemocytes per larva

<sup>b</sup>Not detected.



The plasmatocytes were the largest of the adhering cell types (Table I). Freely-suspended plasmatocytes were oval. Cells contacting the substratum spread over the surface becoming conspicuously fan-shaped in appearance and revealing granular cytoplasm and a ruffled membrane (Plate 1, Figs. 2, arrow; 3; 4, double arrows). Plasmatocytes were capable of movement in the direction of the ruffled membrane. Non-fan-shaped plasmatocytes (Plate 1, Fig. 3) with several areas of ruffled membrane were also detected. These hemocytes were readily distinguished from granular cells which possessed numerous protoplasmic protuberances (Plate 2, Fig. 5, arrow).

The prohemocytes (Plate 2, Fig. 6, double arrows; 7, double arrows) were the smallest of the hemocyte types (Table I). The oval prohemocytes possessed a central nucleus surrounded by a thin periphery of cytoplasm. These hemocytes never adhered to glass or other hemocytes.

The spherule cells (Plate 2, Fig. 8, arrow) varied from fusiform to elliptical. Clusters of uniform spherules (diam  $Y=4.8\mu\text{m}\pm0.1\mu\text{m}$ ) occluded the nucleus. The spherule cells did not adhere to other hemocytes.

The oenocytoids were the largest of the free-floating hemocytes (Plate 3, Fig. 9, Table I) and varied between



## Plate 2

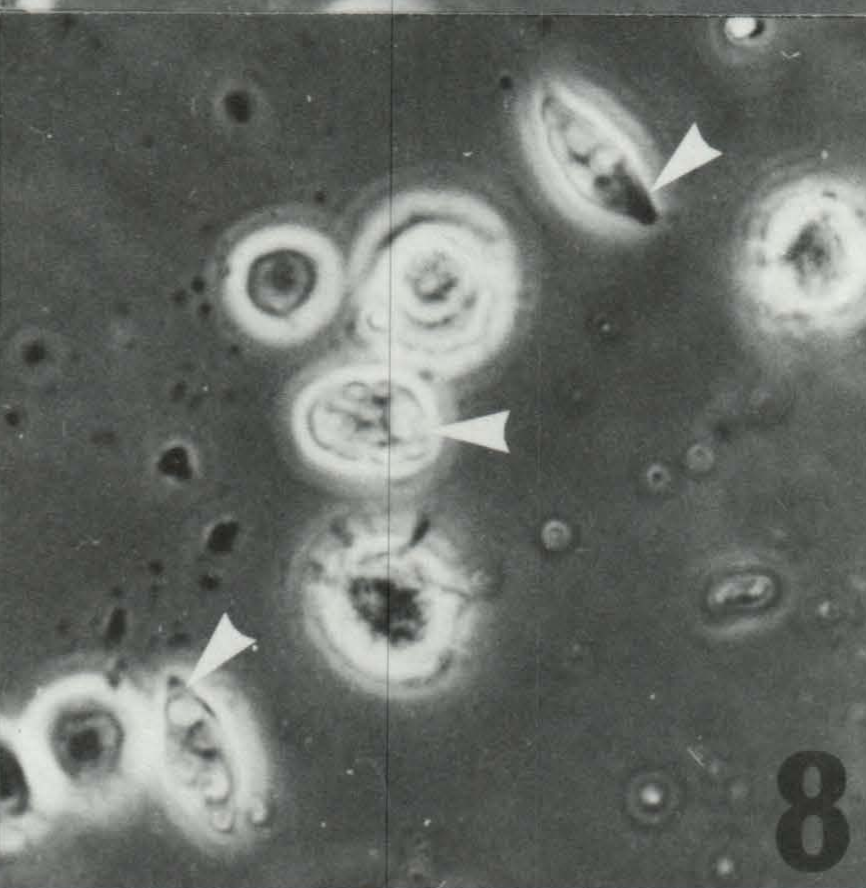
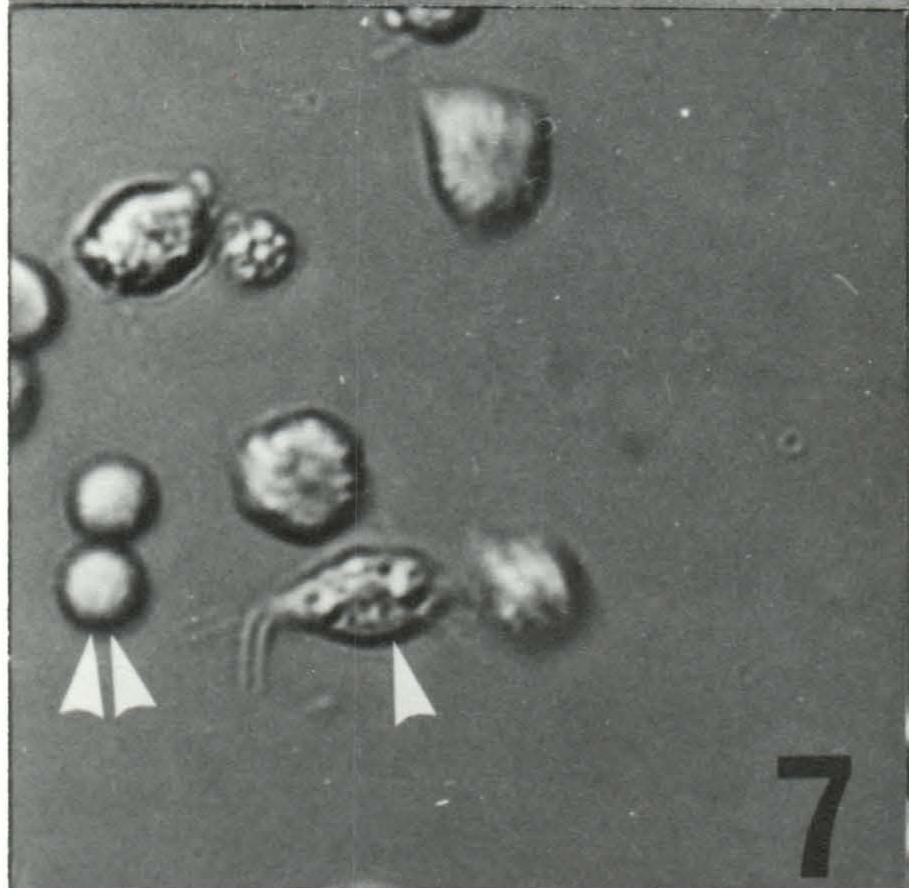
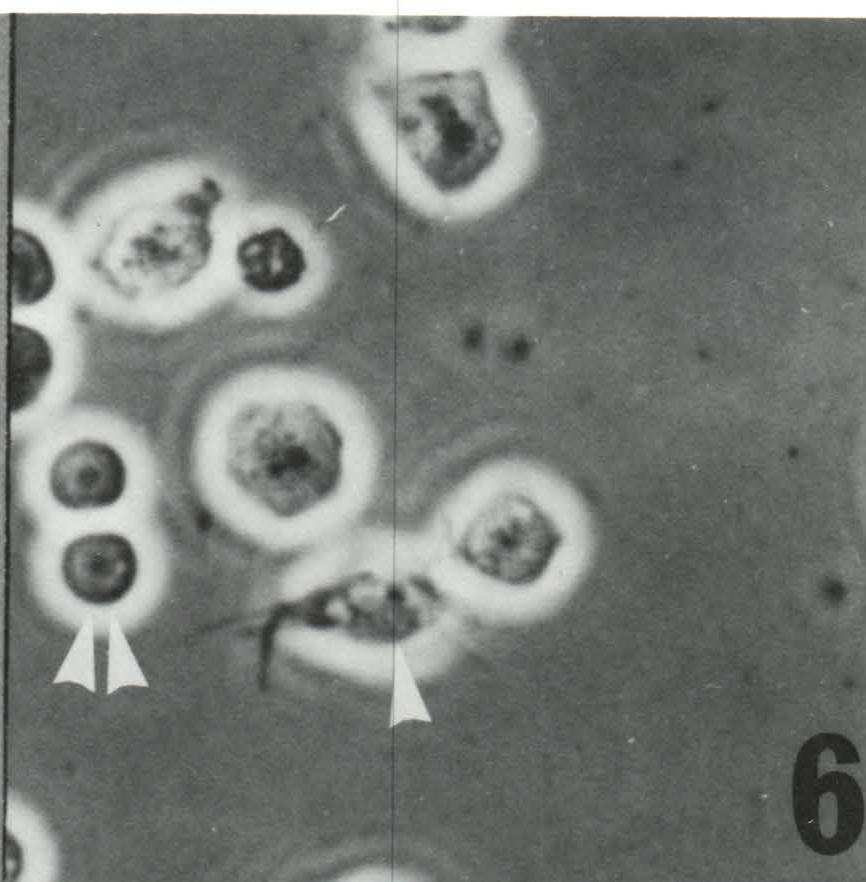
Fig. 5. Granular cell with multiple cytoplasmic projections (arrow) after contact with coverslip for 30 minutes. Phase contrast. X 1300.

Fig. 6. Granular cell (single arrow), prohemocyte (double arrow). Phase contrast. X 800.

Fig. 7 Granular cell (single arrow), prohemocyte (double arrow). Nomarski. X 800.

Fig. 8 Spherule cells (arrows) with intracellular spherules. Phase contrast. X 800.



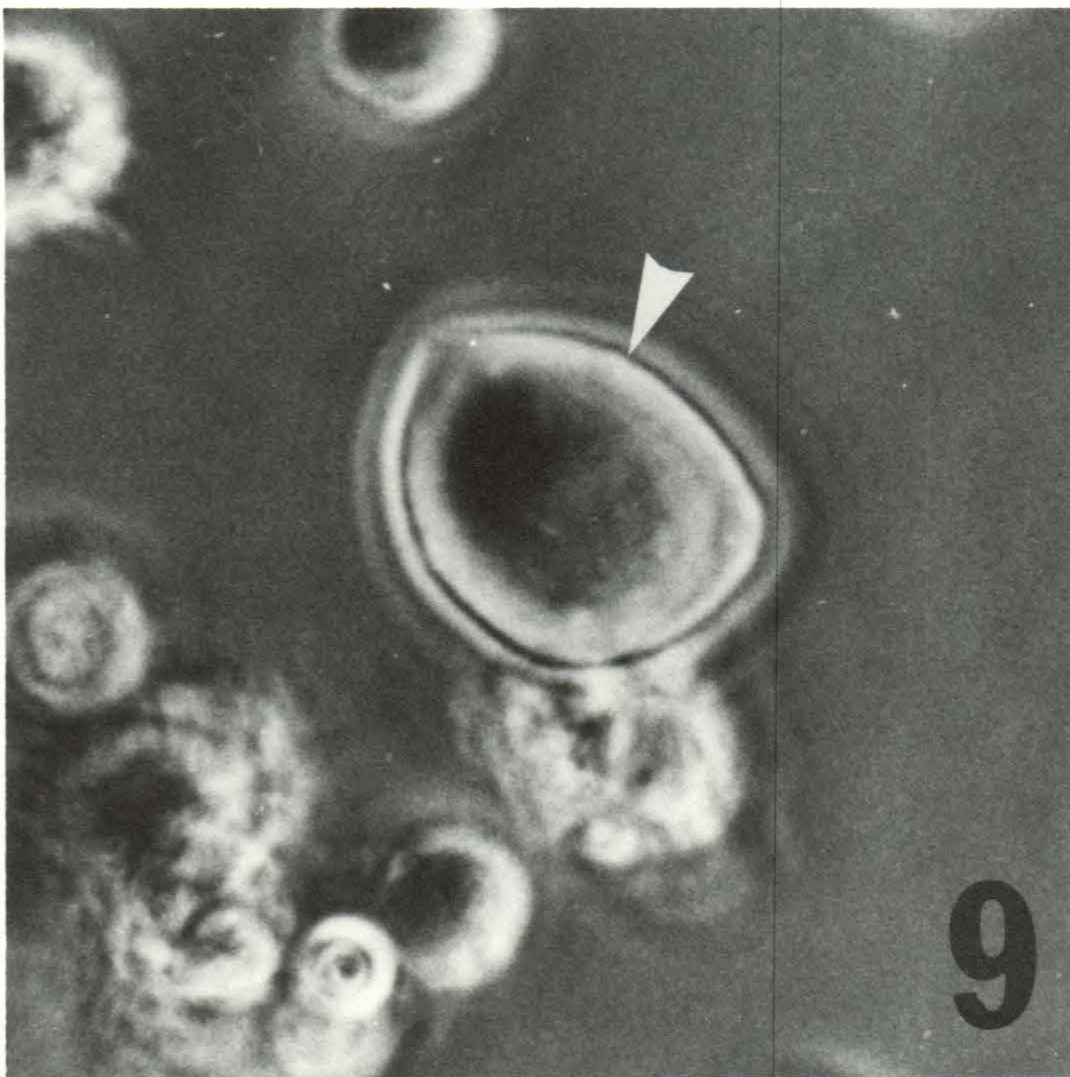




## Plate 3

Fig. 9. Free-floating oenocytoid. Phase contrast.  
X 1100.





9



oval and spherical in shape. An eccentrically located nucleus was detected in hyaline cytoplasm. The oenocytoids occasionally adhered to granular cells.

2. Sexual dimorphism. The total hemocyte counts (THC) of the males increased during the third instar and between successive instars (Table II). The absolute hemocyte counts (AHC) increased exponentially during this period (Table II). Maximum larval THC and AHC levels occurred during the sixth instar. A decrease in THC occurred during the prepupal stage followed by an abrupt increase in early pupation and a 75% decrease in level by late pupation.

The female larvae revealed a bimodal THC profile (Table II) with maximum values in the fourth and sixth instars. The AHC values paralleled those of the THC (Table II). The greatest THC level occurred in the prepupal period followed by a 70% reduction during pupation (Table II).

For both sexes, regardless of the developmental stage, the predominant hemocyte types, in descending order, were the granular cells, plasmatocytes and spherule cells (Tables III and IV).

In the male stages the differential hemocyte counts (DHC) and absolute differential hemocyte counts (ADHC)



Table II  
Total hemocyte and absolute hemocyte counts of several  
postembryonic stages of Choristoneura fumiferana

Sex	Stage of Development	THC <sup>a</sup> (cells/mlX 10 <sup>7</sup> )	Blood Volume (μl)	AHC <sup>b</sup> (cells X 10 <sup>5</sup> /insect)	
Male	Larval	3Le(3)	0.54 ± 0.08	5.0 ± 1.1	0.26
		3L1(8)	1.21 ± 0.29	5.2 ± 0.9	0.63
		4L(12)	1.25 ± 0.14	9.0 ± 0.1	1.12
		5L(22)	1.55 ± 0.18	18.4 ± 4.1	2.85
		6L(30)	2.52 ± 0.38	21.8 ± 2.9	5.49
	Prepupa	(40)	2.04 ± 0.01	—	—
	Pupa, early	(41)	4.47 ± 0.31	—	—
	Pupa, late	(45)	1.45 ± 0.27	—	—
Female	Larval	3Le(3)	0.74 ± 0.03	12.0 ± 1.6	0.88
		4L(12)	1.22 ± 0.03	20.0 ± 1.7	2.40
		5L(22)	0.86 ± 0.01	25.7 ± 2.0	2.21
		6L(30)	1.16 ± 0.13	28.0 ± 1.4	3.25
	Prepupa	(40)	2.87 ± 0.03	—	—
	Pupa, early	(41)	0.97 ± 0.10	—	—
	Pupa, late	(45)	1.06 ± 0.22	—	—

<sup>a</sup>Total hemocyte count, n=25 larvae

<sup>b</sup>Absolute hemocyte count, n=25 larvae

cont.



Table II cont.


<sup>c</sup>First number refers to instar, capital letter to stage, small letter to the stadium, the number in brackets refers to the absolute stadium with reference to 0 day [e.g. 3Le(3), 3rd larval instar, early, 3 days past the first day as a 3L].

<sup>d</sup>Not determined



Table III  
Differential hemocyte counts for Choristoneura fumiferana

Sex	Stage of development		Pr <sup>a</sup>	Pl	G	S	Oe	Ot
Male	Larval	3Le(3) <sup>b</sup>	<sup>c</sup>					
		3L1(8)	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	64.0 $\pm$ 2.4	32.3 $\pm$ 1.8	5.1 $\pm$ 0.7	0.0 $\pm$ 0.0
		4L(12)	8.0 $\pm$ 0.9	21.1 $\pm$ 1.4	44.0 $\pm$ 2.1	7.2 $\pm$ 0.8	16.0 $\pm$ 1.0	9.7 $\pm$ 0.6
		5L(22)	1.1 $\pm$ 0.3	36.0 $\pm$ 1.9	59.4 $\pm$ 2.4	4.0 $\pm$ 0.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
		6L(30)	23.0 $\pm$ 1.5	16.2 $\pm$ 1.3	32.0 $\pm$ 1.8	28.0 $\pm$ 1.7	3.0 $\pm$ 0.5	0.0 $\pm$ 0.0
	Prepupa	(40)	0.0 $\pm$ 0.0	20.0 $\pm$ 1.4	63.8 $\pm$ 2.5	9.4 $\pm$ 1.0	1.5 $\pm$ 0.4	5.3 $\pm$ 0.9
	Pupa, early	(41)	0.0 $\pm$ 0.0	5.1 $\pm$ 0.7	78.0 $\pm$ 2.8	12.0 $\pm$ 1.1	0.0 $\pm$ 0.0	4.9 $\pm$ 1.0
	Pupa, late	(45)	7.1 $\pm$ 0.8	6.0 $\pm$ 0.7	65.3 $\pm$ 2.6	10.7 $\pm$ 1.0	5.3 $\pm$ 0.7	5.6 $\pm$ 1.1
	Female	Larval	3Le(3)	4.0 $\pm$ 0.6	1.0 $\pm$ 0.3	58.2 $\pm$ 2.4	6.0 $\pm$ 0.7	0.0 $\pm$ 0.0
4L(12)								
5L(22)			2.2 $\pm$ 0.4	32.1 $\pm$ 1.8	56.5 $\pm$ 2.4	10.0 $\pm$ 1.0	0.0 $\pm$ 0.2	0.0 $\pm$ 0.0
6L(30)			4.5 $\pm$ 0.7	20.0 $\pm$ 1.4	63.5 $\pm$ 2.5	10.0 $\pm$ 1.0	4.0 $\pm$ 0.6	0.2 $\pm$ 0.9
Prepupa		(40)	0.0 $\pm$ 0.0	14.2 $\pm$ 2.0	73.0 $\pm$ 2.7	19.3 $\pm$ 1.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Pupa, early		(41)	0.0 $\pm$ 0.0	22.1 $\pm$ 1.5	75.0 $\pm$ 2.7	3.0 $\pm$ 0.6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Pupa, late		(45)	0.0 $\pm$ 0.0	20.7 $\pm$ 1.4	70.5 $\pm$ 2.6	9.0 $\pm$ 1.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

<sup>a</sup>Pr = Prohemocyte, Pl = Plasmatocyte, G = Granular cell, S = Spherule cell, Oe = Oenocytoid, Ot =  other or unidentified, intermediate cell type

<sup>b</sup>For code refer to Table II, n=55 larvae, 100 hemocytes/larva

<sup>c</sup>Not determined



Table IV  
Absolute differential hemocyte counts  
for Choristoneura fumiferana

Sex	Stage of Development	Number of cells x 10 <sup>5</sup> /larva						
			Pr <sup>a</sup>	Pl	G	S	Oe	Ot
Male	Larval	3Le(3) <sup>b</sup>	<u>c</u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
		3L1(8)	0.00	0.00	0.40	0.20	0.03	0.00
		4L(12)	0.09	0.24	0.49	0.08	0.11	0.11
		5L(22)	0.03	1.03	1.69	0.11	0.00	0.00
		6L(30)	1.26	0.89	1.76	1.54	0.16	0.00
	Prepupa	(40)	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
	Pupa, early	(41)	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
	Pupa, late	(45)	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
	Female	Larval	3Le(3)	0.04	0.01	0.51	0.05	0.00
4L(12)			<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
5L(22)			0.05	0.71	1.25	0.22	0.00	0.00
6L(30)			0.15	0.65	2.04	0.32	0.13	0.00
Prepupa		(40)	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
Pupa, early		(41)	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>
Pupa, late		(45)	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>	<u>      </u>

<sup>a</sup>For code refer to Table III

cont.



Table IV cont.

<sup>b</sup>For code refer to Table II

<sup>c</sup>Not determined



revealed that the plasmatocytes increased up to the fifth instar (Tables III and IV) and decreased during the last instar. There was a slight increase in level in the prepupal phase followed by a precipitous decline during pupation (Table III). In female spruce budworm the levels of plasmatocytes declined throughout larval development achieving the lowest value during the prepupal phase (Table III). During pupation the incidence of plasmatocytes increased to a constant level. The female larvae had lower ADHC levels for the plasmatocytes than did the male larvae (Table IV). The male pupae had lower DHC levels for the plasmatocytes than did the female pupae (Table III).

The level of granular cells in the males changed significantly between different instars with an overall trend of decreasing values during the larval instars (Table III, IV). The levels increased during prepupation and early pupation to 78% followed by a decline in late pupation. The sixth instar female larvae had higher ADHC values for the granular cells than the males (Table IV). The spherule cells in the male spruce budworm were lowest in the fourth and fifth larval instars and essentially leveled off during the prepupal and pupal periods (Table III). In the females the lowest levels occurred during pupation (Table III). The females had higher DHC levels in the fifth instar and prepupal stage than the males and had higher ADHC values



in the fifth and sixth larval stage (Table III, IV).



## B. Discussion

The five types of hemocytes found in the spruce budworm, including mobile plasmatocytes, have been reported for other species of Lepidoptera (see Price and Ratcliffe 1974).

Because of differences in the species studied, the stage of development, physiological status and the bleeding and fixing techniques, comparisons amongst studies is difficult (Shapiro 1979). However, the phenomenon of increasing THC and AHC in successive spruce budworm larval stages is similar to hemocyte changes found during the larval growth of Ephestia kühniella Zell. (Arnold 1952) and Pieris brassicae L. (Breugnon and LeBerre 1976). The peaking of the hemocytes of the last larval stage of the male spruce budworm and the decline during prepupation is similar to results found for E. kühniella (Arnold 1952), Bombyx mori L. (Nittono 1960), G. melonella (Shrivastava and Richards 1965), Papilio demoleus L. (Narayanan and Jayaraj 1976) and L. fiscellaria fiscellaria (Boiteau and Perron 1977). Unlike the results of numerous studies of insect hemocytes, the hemocytes in the male spruce budworm increased again during pupation. The results for the female spruce budworm are also unique in that the hemocyte level peaked during the prepupal stage and declined there-



after. The present results establish that the stage in which the THC culminated was dependent on the sex of the spruce budworm.

The reason for the increase in THC and AHC throughout the development of the third male instar in view of a constant hemolymph volume is not known.

The decline in the plasmatocytes and increase in granular cells with culmination of the latter prior to pupation in the female spruce budworm is similar to results for Spodoptera eridania (Yeager 1945) and P. brassicae (Misselunene 1965). The results of Boiteau and Perron (1977), reveal that the granular cells increase in level as in the present results. The plasmatocytes also increase in level which was not the case in the spruce budworm. The culmination of granular cells during early pupation among male spruce budworm is similar to the results in G. mellonella (Shrivastava and Richards 1965) and the three saturniids Antheraea polyphemus (Cramer), Samia cynthia Drury and Hyalophora cecropia (L.) (Walters 1970). The increase in granular cells of spruce budworm may be reflective of preparation for pupal-adult transformation similar to that reported by Whitten (1964, 1969) and Zachary and Hoffmann (1973) for cyclorrhaphous dipterans. The reduction in granular cells during pupation of the male spruce budworm is comparable to that of



L. fiscellaria fiscellaria (Boiteau and Perron 1977).

The maximum levels of spherule cells in the spruce budworm, depending on the sex, occurred either during the last instar or in the prepupal phase followed by a decline during pupation. This was similar to the change in E. kuhniella reported by Arnold (1952) and may be associated with changes in lipid metabolism. The present results paralleled those of Boiteau and Perron (1977) if the Price and Ratcliffe scheme (1974) is applied.

Changes in DHC profiles during insect development are common (Arnold 1974, Jones 1967a,b,c, Breugnon and LeBerre 1976, Boiteau and Perron 1977) and appear to be under endocrine control (Judy and Marks 1971, Takeda 1977).

One interesting result of the present work was the detection of quantitative and temporal THC, AHC, DHC and ADHC sexual dimorphism. Hemocyte sexual dimorphism has been previously detected in adult stages of Periplaneta americana L. (Smith 1938), Mantis spp. and Ameles spp. (Avry et al. 1949) and Locusta migratoria migratorioides Reiche and Fairmaire (Webley 1951). The significance of the present dimorphism remains undetermined.



## II. In vivo and in vitro hemolymph experiments.

### A. Labdina fiscellaria fiscellaria

#### 1. Results.

(i) In vivo injection results. After 72h incubation, protoplasts were still detected only in protoplast-injected larvae. The pleomorphic protoplasts (Plate 4, Fig. 1, single arrow) were larger than the hemocytes (Plate 4, Fig. 1, double arrows). Many of the protoplasts had short tapering processes (Plate 4, Fig. 2, arrow) and long processes. The protoplasts were actively mobile and moved both the main cellular mass and the protoplasmic extensions. During this activity, the protoplasts often collided with the spherule cells, plasmatocytes and granular hemocytes. These types of hemocytes were never detected adhering to the protoplasts.

After 168h, conidiophores bearing conidia characteristic of E. egressa were observed on the dead larvae in the protoplast-injected group. There were no mortalities in the control larvae.

#### (ii) In vitro experiments.

(a) Protoplasts. The McCT method was



used to repeat in in vivo protoplast-hemocyte experiment. Initially, the fungal cells in the control protoplast suspensions and hemolymph-protoplast mixture were quite active with the filopodial-like protoplasmic extensions moving rapidly. With time, fewer protoplasts with extensions were detected in the control groups ( $12.6\% \pm 0.1\%$ ,  $n = 100$ ) than in the hemolymph treated protoplasts ( $84.8\% \pm 1.7\%$ ,  $n = 100$ ). The extensions in the control samples ( $18.3 \mu\text{m} \pm 1.1 \mu\text{m}$ ,  $n = 100$ ) were significantly shorter than those in the treated samples ( $92.6 \mu\text{m} \pm 2.1 \mu\text{m}$ ,  $n = 100$ ;  $P < 0.001$ ). The protoplasts did not adhere to any of the hemocyte types. The protoplasmic extensions were often seen contacting the spherule cells (Plate 4, Fig. 3, arrow) and wrapping around the spherule cells. The extensions also made contact with the granular hemocytes (Plate 4, Fig. 4). Whenever the protoplasts abutted the hemocytes, two distinct cell types were clearly visible: fungal cells (Figs. 5, 6 arrow) and insect hemocytes (Plate 4, Figs. 5, 6 double arrows). Granular cells were attached to the nylon fibers in both the hemocyte control samples and the protoplasts plus hemocytes samples in equal numbers,  $8 \pm 2$  granular cells/fiber and  $9 \pm 1$  granular cells/fiber ( $t = 0.447$ ,  $P < 0.5$ ), respectively.

(b) Bacteria. Plasmatocytes were detected with E. coli cells on the ruffled membrane



(Plate 5, Fig. 7) and on the general surface of these hemocytes (Fig. 8). The granular hemocytes contained the majority of the E. coli cells (Table V, Plate 5, Fig. 9); while the spherule cells were free of test particles (Plate 5, Fig. 10). The MccT method significantly ( $P < 0.001$ ) increased the level of E. coli adhesion to the granular cells above that of the direct monolayer method by a factor of 2 (Table V) but the incidence of phagocytosis was low (0.1%). The granular cells were often detected forming large aggregates with the test particles. Such aggregates were rare in the control samples.

(c) Sporangiospores of *A. repens*.

The granular hemocytes had  $2.9 \pm 0.3$  spores/hemocyte adhering to the hemocyte surface (Table V, Plate 5, Fig. 11). The other types of hemocytes did not interact with the spores. There were no signs of nodulation. The granular cells with spores were not believed to have been confused with possible hemocyte-spherule cell transformations because the spore dimensions and morphology bore no resemblance to the spherules from the spherule cells and the control hemocyte cultures (Plate 5, Fig. 12; plasmatocytes, arrow; granular cells, double arrows) did not reveal such transformations.







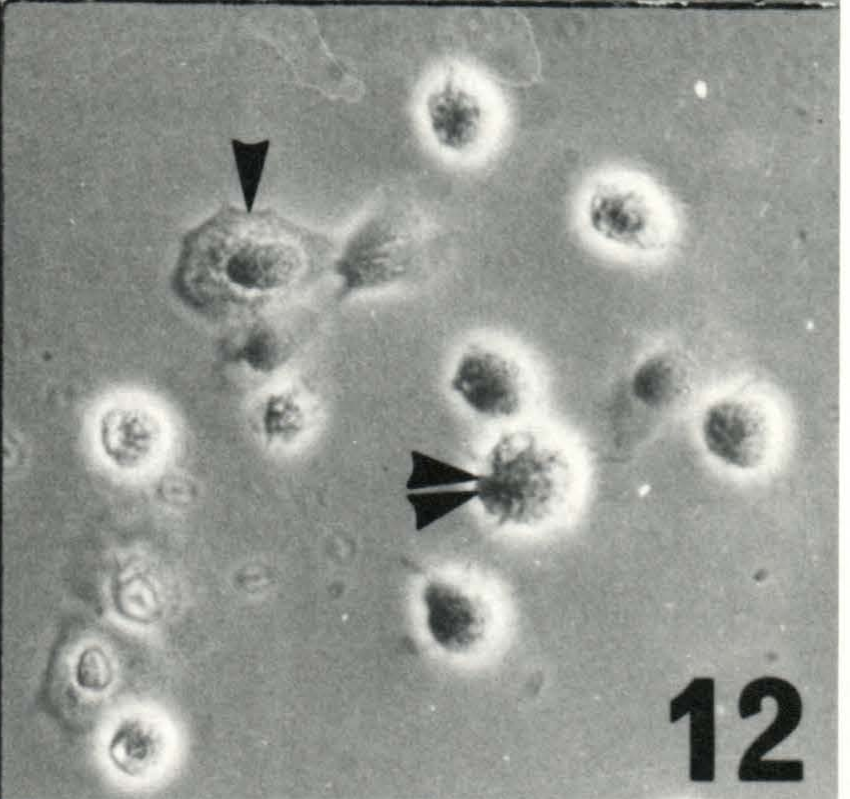
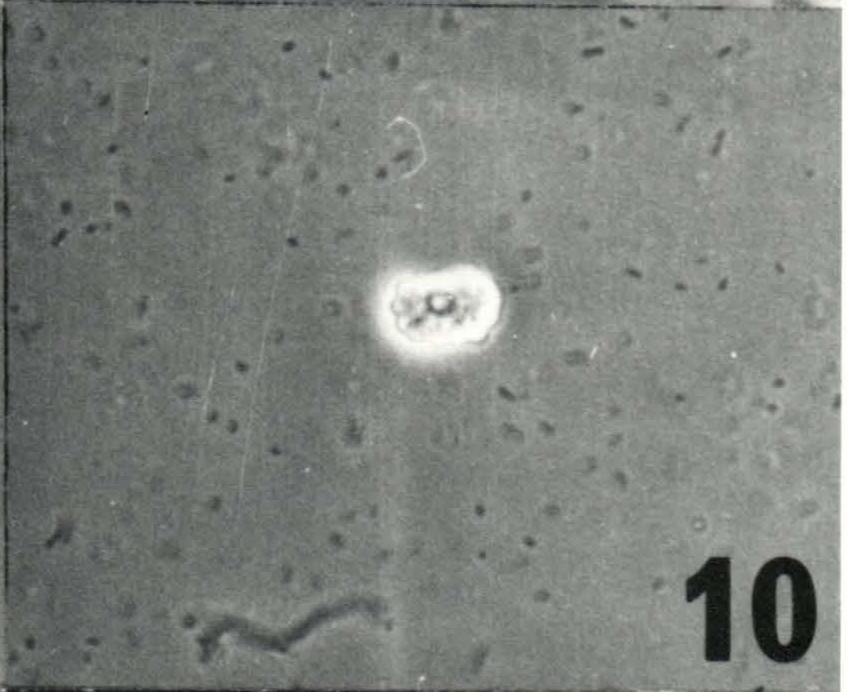
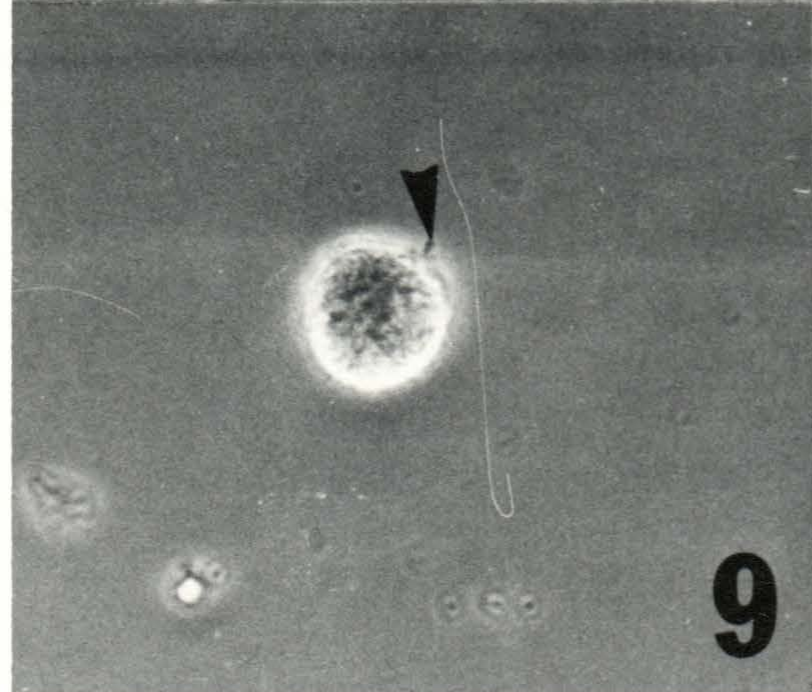
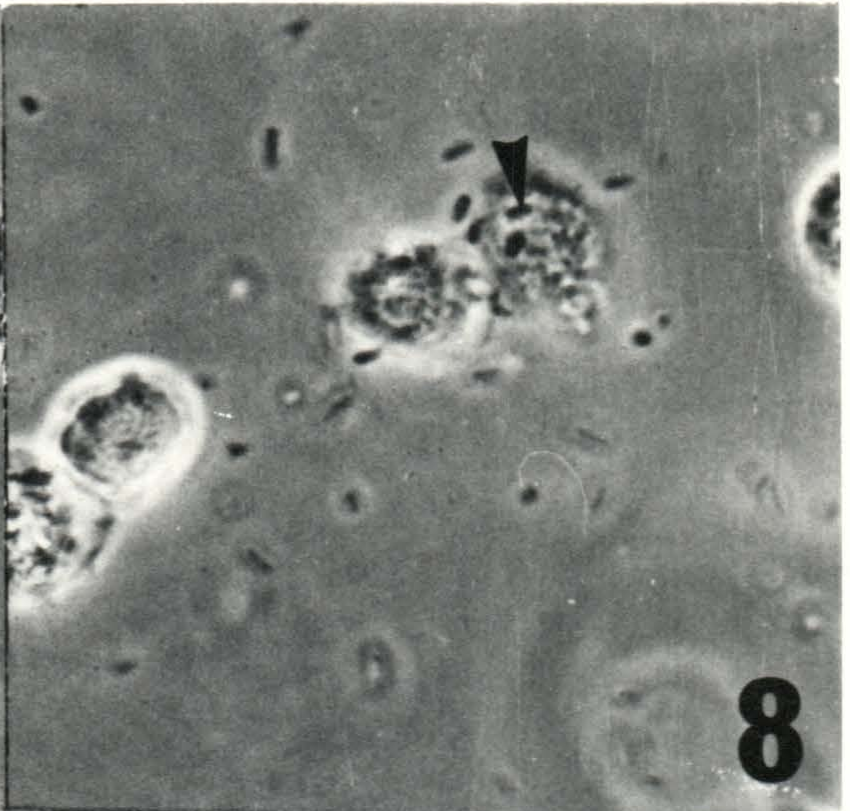




Table V

Interactions between Escherichia coli cells and Absidia repens sporangiospores and the hemocytes of Labdina fiscellaria fiscellaria

Test organism	Type of hemocyte	Particles/hemocyte <sup>a</sup>	% of hemocytes with particles <sup>a</sup>
<u>E. coli</u> (mono-layer method)	Granular hemocytes	1.9 $\pm$ 0.1 (n=270)	24 $\pm$ 1 (n=95)
	Spherule cells	0.0 $\pm$ 0.0 (n=250)	0 $\pm$ 0 (n=100)
<u>E. coli</u> (MCCT) <sup>b</sup>	Granular hemocytes	4.4 $\pm$ 0.6 (n=400)	75 $\pm$ 4 (n=50)
	Spherule cells	0.0 $\pm$ 0.0 (n=350)	0 $\pm$ 0 (n=100)
<u>A. repens</u> (MCCT)	Granular cells	2.9 $\pm$ 0.3 (n=500)	68 $\pm$ 5 (n=75)
	Spherule cells	0.0 $\pm$ 0.0 (n=500)	0 $\pm$ 0 (n=100)

<sup>a</sup>Values represent Mean  $\pm$  standard error, n is sample size.

<sup>b</sup>Mcct, Microcapacity centrifuge tube method.



(d) Hyphae of *Rhizopus nigricans*.

It was not feasible to quantify the interaction between the hemocytes and the hyphae of *R. nigricans*. The granular cells were observed adhering to and flattening on the surface of these hyphal walls as either single hemocytes (Plate 6, Fig. 13, arrow) or in aggregates of hemocytes (Plate 6, Fig. 13, double arrows). During the movement of nondiluted hemolymph streams past the hyphae, it was noticed that once the granular cells adhered to the hyphae other granular cells readily adhered to the interacting hemocytes favouring the development of large melanotic masses about the hyphae (Plate 6, Fig. 14, arrow). Encapsulation occurred over the entire hyphal surface including the growing hyphal tips (Plate 6, Fig. 15).

Within 30 min of incubation on the slide, many of the single hemocytes and most of the peripheral hemocytes in the capsules commenced moving. A fine filament was left behind the mobile hemocytes on the hyphal walls. After incubation at 12°C for 24h, extensive filament formation was detected (Plate 6, Fig. 16, arrows) with many filaments linking and crossing over each other. Only the granular cells were involved.

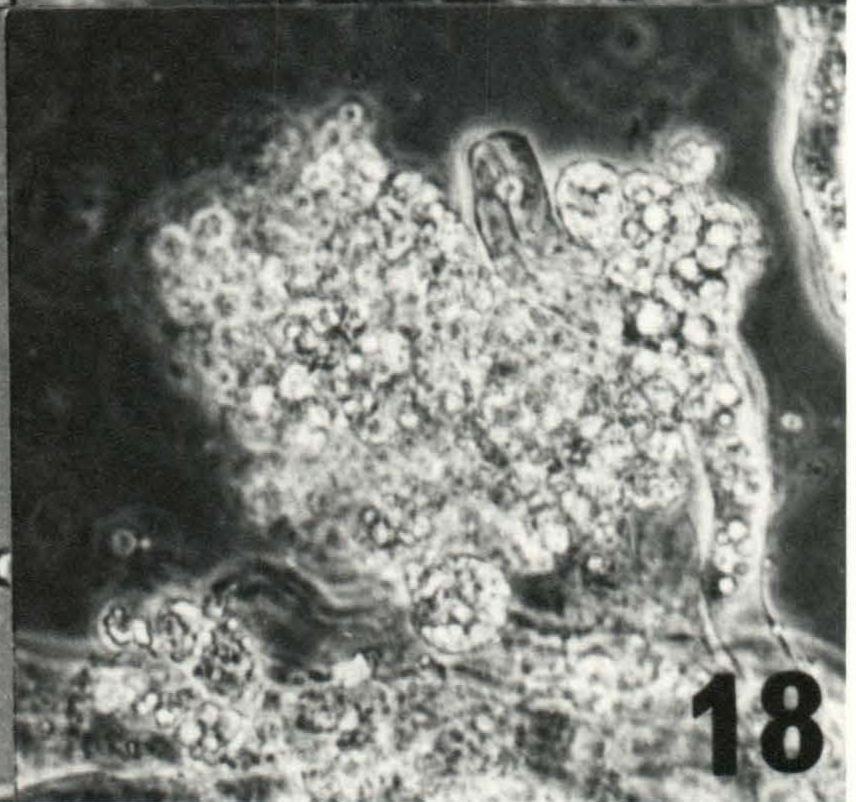
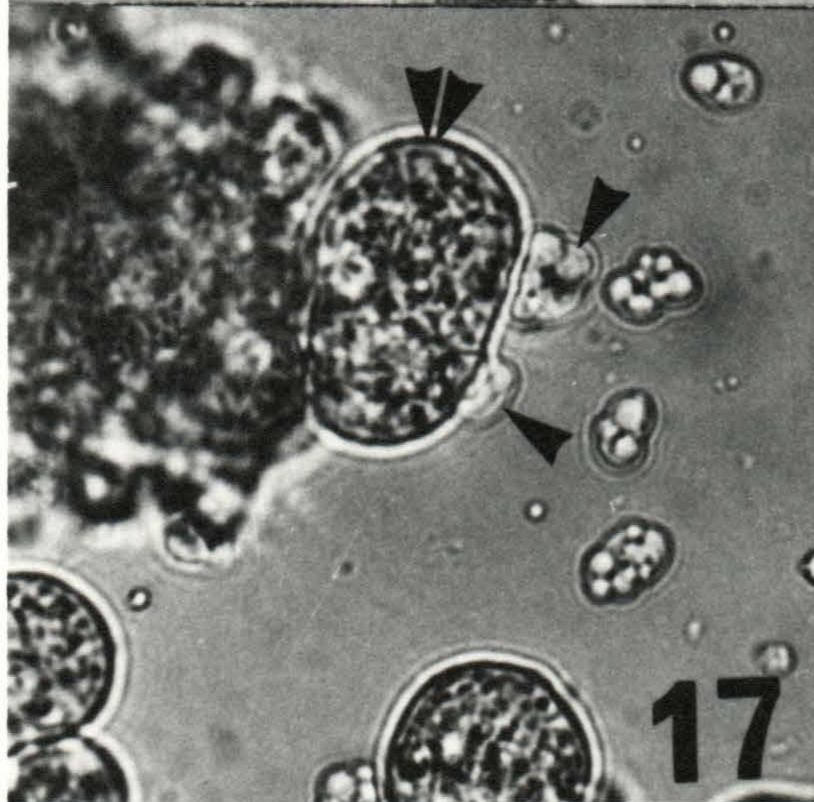
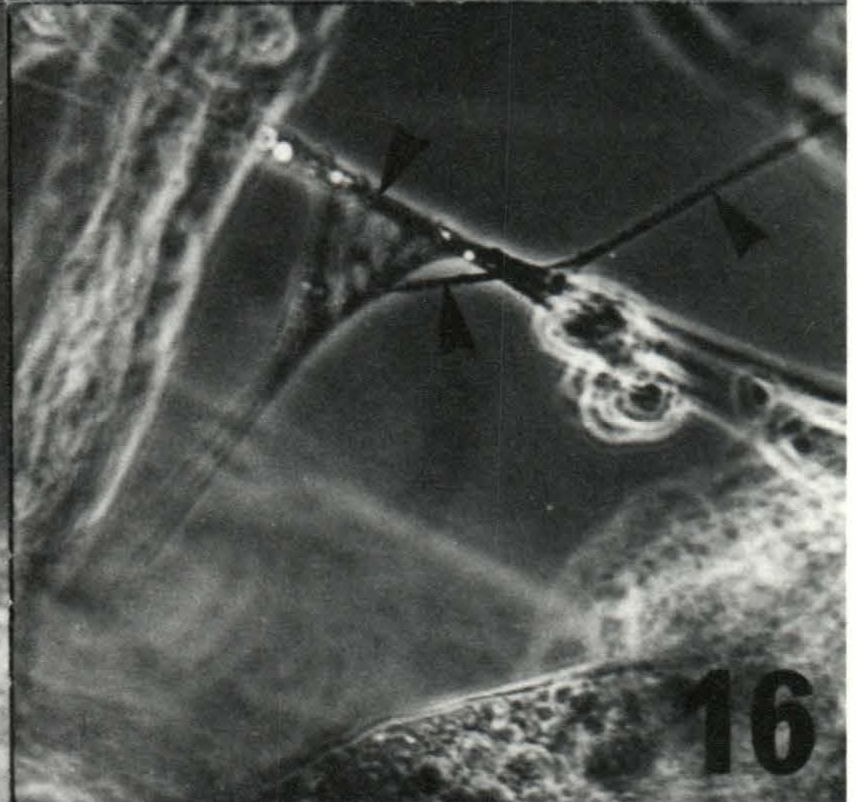
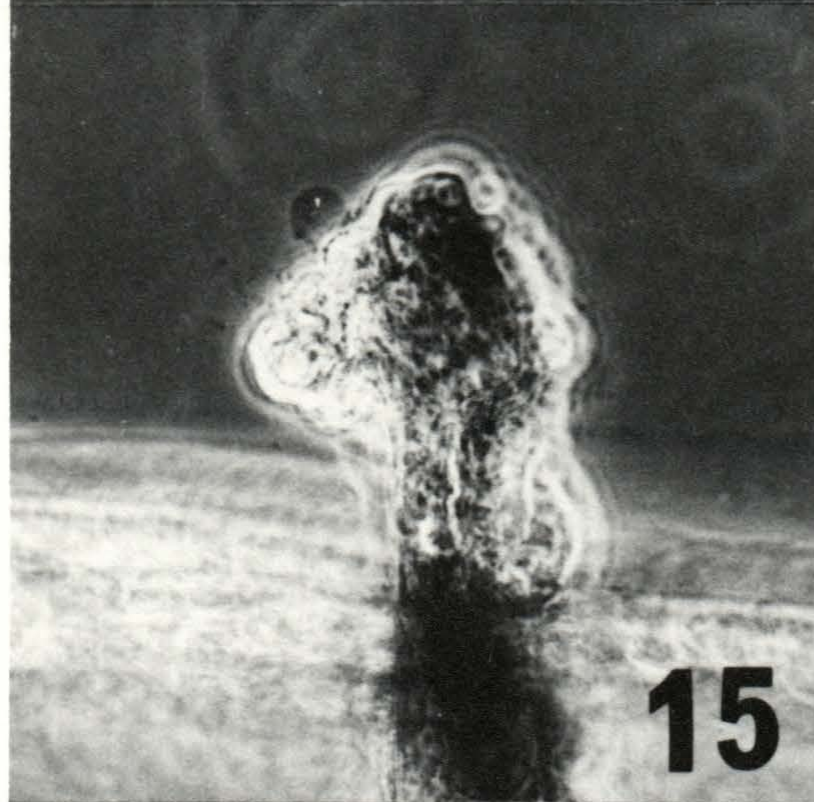
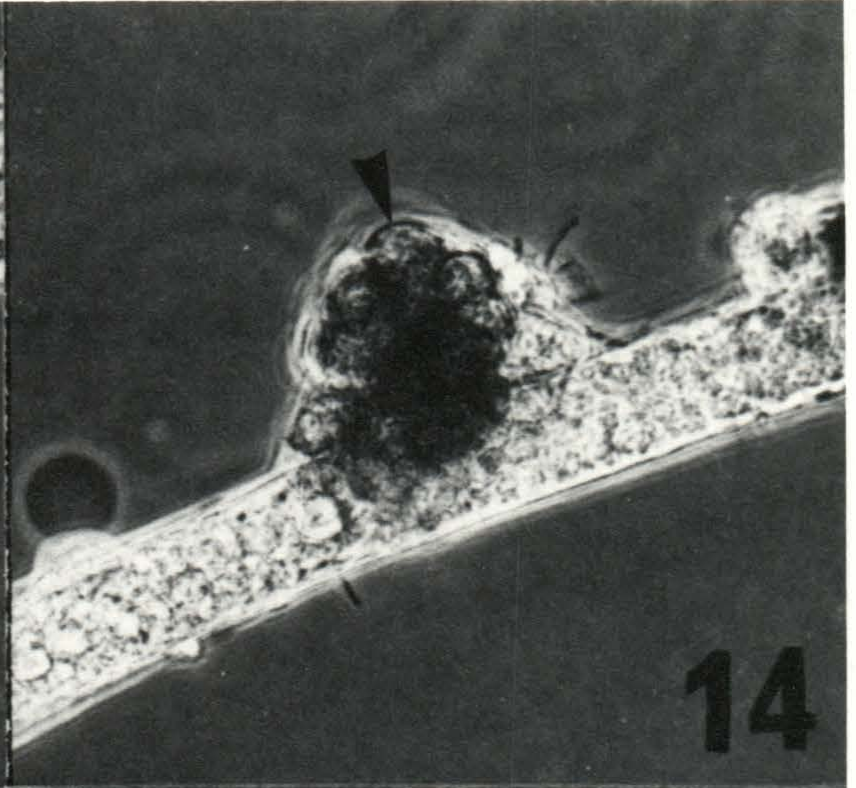
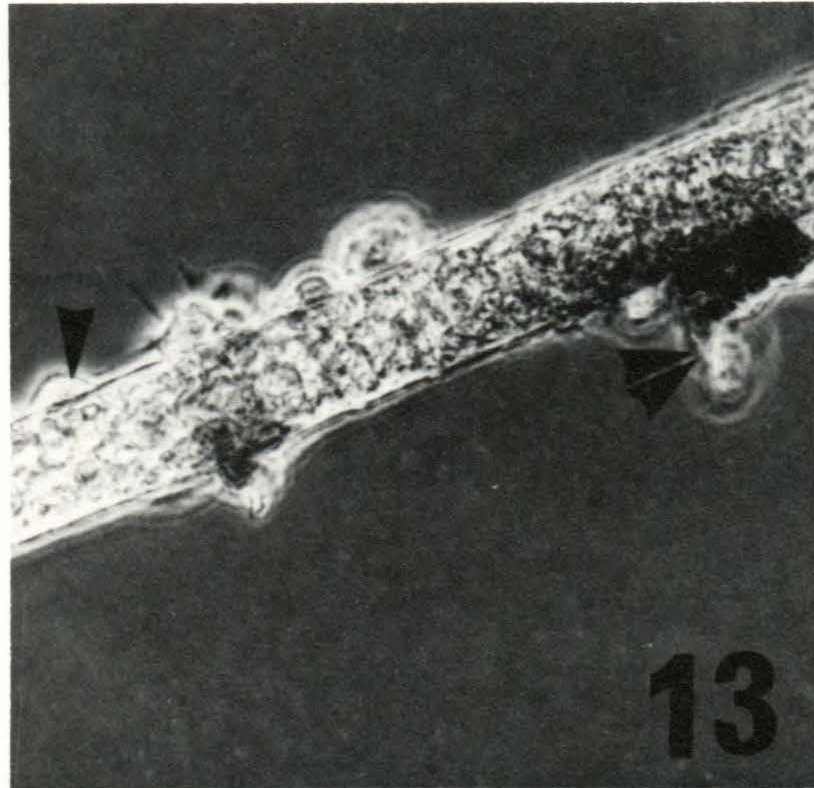
(e) Walled stages of *Entomophthora egressa*. The spherule cells were observed adhering to



## Plate 6

- Fig. 13. The adhesion of single hemocytes (arrow) and aggregates of hemocytes (double arrows) to a hypha of Rhizopus nigricans. Phase contrast. In vitro. ML. X 540.
- Fig. 14. Melanotic hemocyte mass (arrow) on a hypha of Rhizopus nigricans. Phase contrast. In vitro. ML. X 540.
- Fig. 15. Capsule surrounding the hyphal tip of Rhizopus nigricans. Phase contrast. In vitro. ML. X 540.
- Fig. 16. Filaments (arrow) formed by the granular cells. Phase contrast. In vitro. ML. X 540.
- Fig. 17. Spherule cells (arrows) adhering to a hyphal body (double arrow) of Entomophthora egressa. Phase contrast. In vitro. MccT. X 630.
- Fig. 18. Spherule cells encircling the hyphal stage of Entomophthora egressa. Phase contrast. In vitro. MccT. X 650.







both the hyphal body stage (Plate 6, Fig. 17; spherule cell, single arrow, hyphal body, double arrow) and the hyphal stage (Plate 6, Fig. 18) of E. egressa. The adhesion of spherule cells to the hyphal bodies was not as extensive as that on the hyphae. On the latter, the spherules formed a loose irregular aggregate. The spherule cells lysed upon contact with the hyphae, releasing the spherules. No melanization was observed. After incubation at 12°C for 24h there were no filamentous connections between hyphal segments.

(f) Sephadex bead experiments. Proto-plasts were found adhering to only the positively charged DEAE-Sephadex beads (pH4.2) (Plate 7, Figs. 19, 20, Table VI) and readily disassociated from these beads when the charge was neutralized. The hemocytes also adhered to positively-charged DEAE beads (Plate 7, Fig. 21, Table VI). The adhesion of the first few hemocytes appeared to enhance the adhesion of additional hemocytes resulting in a near encapsulation of the beads. It was found that only the charged DEAE beads attracted hyphae (Plate 7, Fig. 22, arrows), conidia (Plate 7, Fig. 23) and seemingly empty hyphal cell walls (Plate 7, Fig. 24) of E. egressa. These stages were released from the beads by negating the positive charge by raising the pH to 7.2. These test particles were not attracted to either neutral (pH4.2) or negatively-charged



CM-Sephadex beads (pH7.2).



Table VI  
Interaction between Entomophthora egressa protoplasts  
and hemocytes and charged ion exchange resins

Test particle	Type of resin	Particles/bead <sup>a</sup>	% of beads with particles <sup>a</sup>
Protoplasts	DEAE-Sephadex	3.2 $\pm$ 0.3 (n=40)	73 $\pm$ 7 (n=100)
	CM-Sephadex <sup>d</sup>	0.0 $\pm$ 0.0 (n=40)	0 $\pm$ 0 (n=100)
Hemocytes	DEAE-Sephadex	<sup>b</sup>	100 $\pm$ 0 (n=100)
	CM-Sephadex	0.0 $\pm$ 0.0 (n=50)	0 $\pm$ 0 (n=100)

<sup>a</sup>values represent Mean  $\pm$  standard error, n equals sample size

<sup>b</sup>not determined because of massise aggregation of the test particles about the DEAE-Sephadex resin.

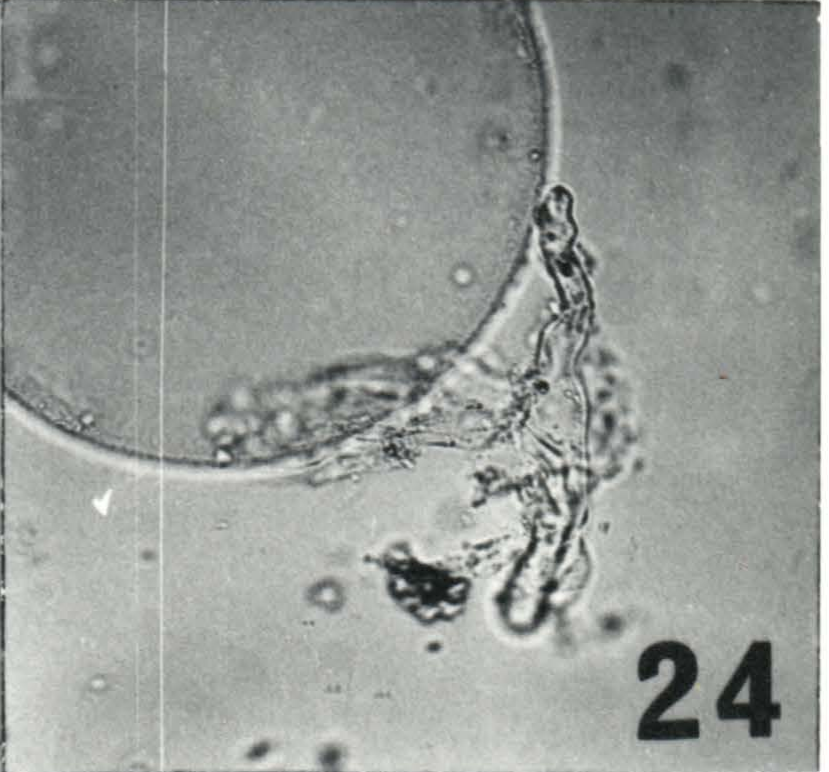
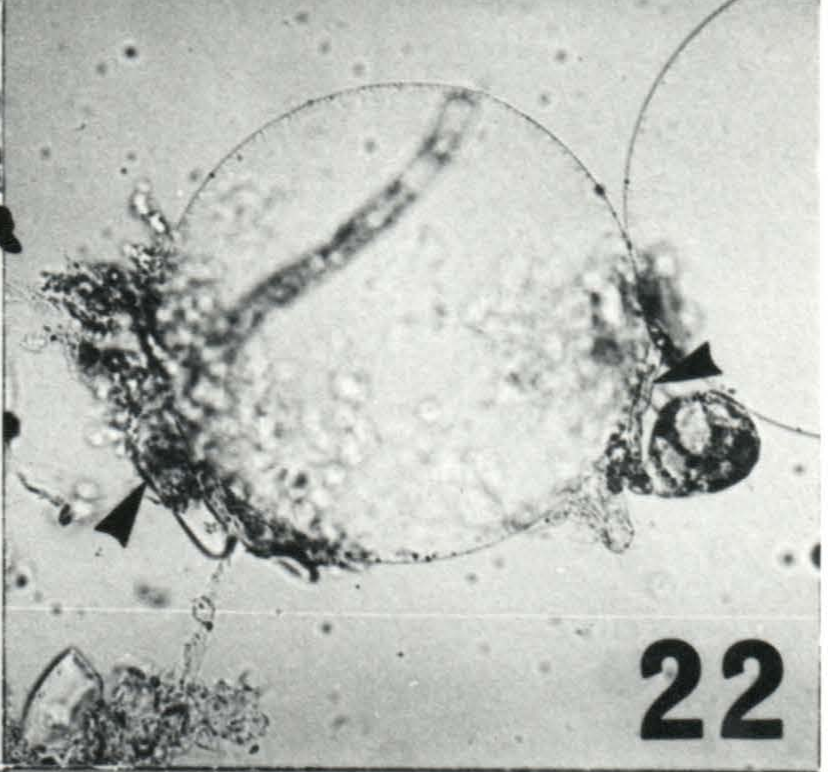
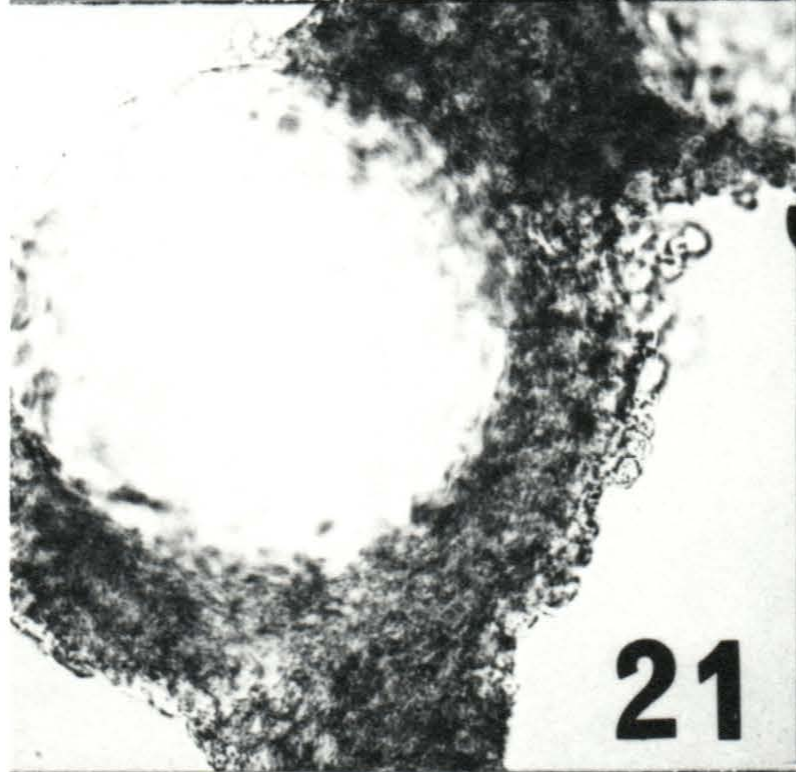
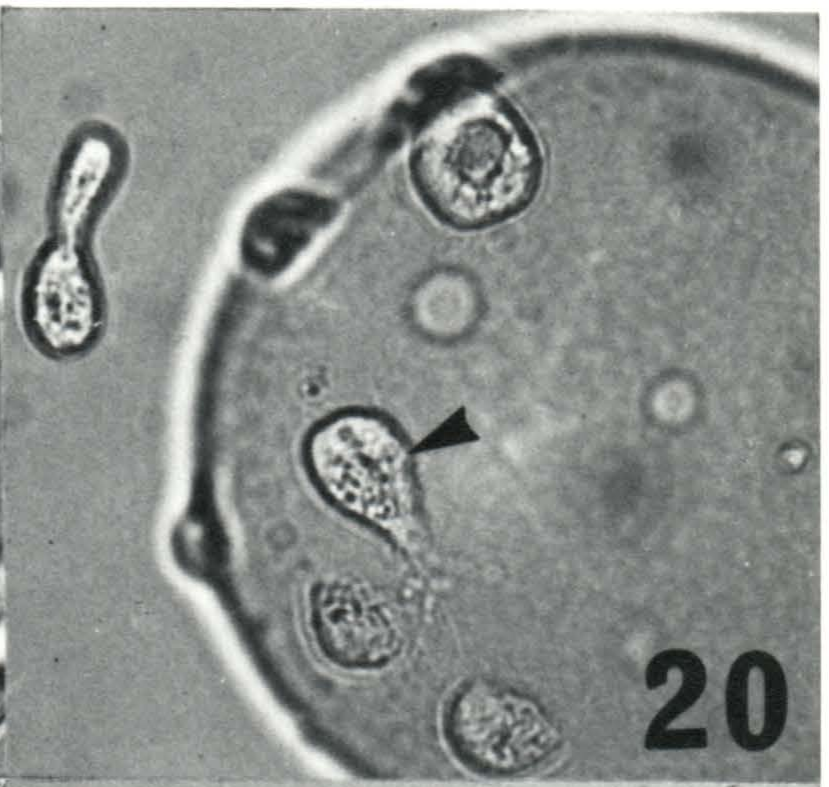
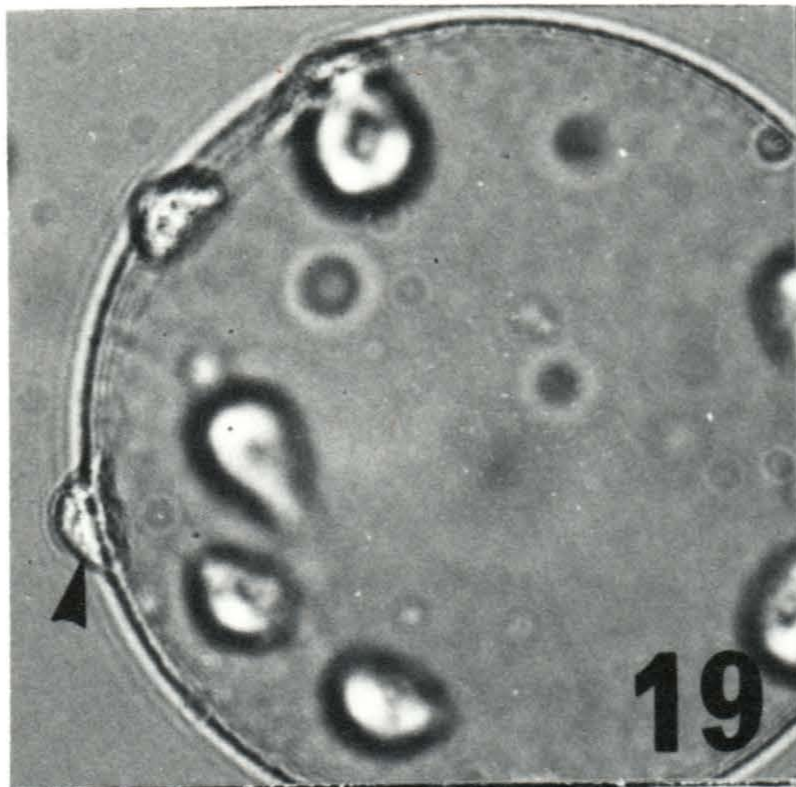
<sup>c</sup>positively charged beads at pH4.2

<sup>d</sup>negatively charged beads at pH7.2











## 2. Discussion.

The presence of mobile free-protoplasts of E. egressa with active protoplasmic extensions in the hemolymph of L. fiscellaria fiscellaria after 72h post-injection and the development of conidia characteristic of E. egressa would suggest that the protoplast stage is a natural portion of the life cycle of E. egressa in the eastern hemlock looper. Tyrrell (1977) has shown that the free-protoplast stage occurs naturally in the eastern spruce budworm, Choristoneura fumiferana (Clem.). It is interesting to note that Tyrrell (1977) used isolates of E. egressa from two different hosts; one from the eastern hemlock looper from Anticosti Island, Quebec, and the other from the eastern spruce budworm from Newfoundland. In both cases 90-100% infections were reported. Tyrrell's photomicrographs indicated no obvious physical interaction between the protoplasts and the host hemocytes. This would imply that the eastern spruce budworm has little, if any, cellular immune response to the protoplasts from the different hosts. The hemlock looper also lacks a physical cellular response to protoplasts of I458 of E. egressa.

The absence of hemocytic responses to the pathogens suggested that the eastern hemlock looper and the eastern spruce budworm appear to be the habitual hosts of E. egressa.



The nonrandom, prolonged contact of the protoplasmic extensions with the spherule cells and the granular hemocytes implies a possible chemosensory role for these tapering processes. The increased length of the protoplasmic extensions on the hemolymph treated protoplasts in the in vitro experiments compared to those of the control protoplasts may either represent a nutritional response or a response resulting from possible anti-protoplast activity in the hemolymph. This aspect will be considered in greater detail in section II. B. 2.

The interaction of the granular cells with E. coli, A. repens, and R. nigricans suggests that these hemocytes may play an important role in the immune responses of the eastern hemlock looper. Ratcliffe and Rowley (1979) proposed that the granular cells are vital to the recognition of non-self particles. Phagocytic granular cells have been reported for Spodoptera eridenia (Cramer) (Yeager 1945), Pieris rapae crucivora Boisduval (Takada and Kitano 1971) and Calpodes ethlius Stoll (Neuwirth 1974). Ratcliffe and Rowley (1974, 1975) have assessed the significance of phagocytosis in several species of Lepidoptera and found that the granular cells of G. mellonella and Pieris brassicae (L.) have some phagocytic activity. However, plasmatocytes are generally believed to have the major phagocytic role in most insects (Wittig 1965, Ratcliffe and Rowley 1975, 1979).



The nature of the test particles affected the type of hemocytic response in the present study. Suspensions of granular hemocytes and E. coli initiated nodulation. Similar activity has been reported for the plasmatocytes and granular cells of P. brassicae, Calliphora erythrocephala (Meig.), P. americana and G. mellonella (Ratcliffe and Rowley 1975). When spores of A. repens were the test particles, such hemocyte activity was not detected. Vey and Vago (1969) and Vey (1969) described the initiation of nodulation in vivo and in vitro, respectively, by the plasmatocytes of G. mellonella to the conidia of A. niger after 5-30min incubation. These differences may reflect the physicochemical nature of the test particles, the host insect and the incubation procedures and conditions.

The type of hemocyte and the response of these cells to R. nigricans was not like the plasmatocyte encapsulative response of G. mellonella to the hyphae of M. hiemalis (Vey 1968) or to B. bassiana (Vey and Vago 1971). Vey and Farques (1977) have documented the same response by the plasmatocytes of larvae of L. decemlineata to infection by B. bassiana. In the present study, the non-uniform capsule was initiated by the asymmetrical adhesion of hemocytes in a fashion analogous to that detected in the crayfish Astacus astacus L. in response to Aphanomyces astaci Schikora (Unestam and Nylund 1972) and in the molluscs Zonites sp.



and Helix aspersa Muller to M. anisopliae (Vey et al. 1975). The capsules in this study were similar to those of other insects responding to fungi in that the hemocytes formed a melanotic, multilayered mass around R. nigricans. The selective, enhanced adhesiveness of granular cells on the hyphae of R. nigricans implied that a change in the membrane had been induced by the hemocytes contacting the test particles. Metarhizium anisopliae induced a similar response by the hemocytes of Zonites sp. and H. aspersa (Vey et al. 1975). Salt (1970) has speculated on the possible involvement of changes on hemocyte surfaces as a means of controlling the encapsulation of insect parasitoids and Ratcliffe and Rowley (1979) have argued strongly in favour of this. The role of melanization in the hemocyte responses to the test particles appears to be ambiguous, a view currently shared by others (Nappi 1975, Soderhall 1978). In keeping with the Price and Ratcliffe (1974) scheme, in which it was suggested that all ameboid hemocytes capable of motion in vitro be designated as plasmatocytes, it would appear that the granular cells of the present study were transformed into plasmatocytes during contact with the hyphae. Developmental interrelationships between various types of hemocytes have been considered by Lai-Fook (1973) and Price and Ratcliffe (1974).



Having established that the granular cells play a significant role in in vitro hemocyte immune response to a variety of test particles, it was interesting to note that the spherule cells were very active against hyphal bodies and hyphae of E. egressa in vitro. Reik (1968) suggested that the spherule cells of insects secrete adhesive acid mucopolysaccharides on the periphery of developing cellular capsules eliciting the accretion of additional hemocytes. Spherule cells have been implicated in a similar role in the in vitro formation of nodules of Lepidoptera (Ratcliffe and Rowley 1975). Ratcliffe (1975) has speculated that the induction of stress in the spherule cells or closely allied granular cells may be a factor involved in the recognition of foreignness by insects. It is not known why the spherule cells and not the granular hemocytes of the eastern hemlock looper reacted to the walled stages of E. egressa.

The surface charge of foreign particles is known to influence phagocytosis (Walters and Williams 1966, Marsot and Couillard 1978). Walters and Williams (1966) reported that the plasmatocytes of the pupae of Anthereae polyphemus (Cramer) and Hyalophora cecropia L. readily adhered to the beads of the anion-exchange DEAE-Sephadex and only sparingly contacted the negatively-charged CM-Sephadex resin beads and that the plasmatocytes failed



to contact the DEAE-Sephadex resin beads when their mobility had been inhibited by ethylenediamine-tetraacetic acid (EDTA) and 2,4-dinitrophenol. This would suggest that the hemocytes were electrostatically induced to migrate toward the DEAE-resin as opposed to being passively attracted by unlike charges. A similar theory has been advanced for the phagocytosis of DEAE-Sephadex beads by Amoeba proteus L. (Marsot and Couillard 1978). Brewer and Vinson (1971) found that EDTA reduced the encapsulative response of Heliothis zea (Boddie) to the eggs of the parasitoid Cardiochiles nigriceps Vierick. They thought that the surface charge on the egg had a role in the process. Vinson (1974) reported that the hemocytes of Heliothis virescens (Fabricius) and H. zea adhered to the DEAE-Sephadex resin and not to the CM-Sephadex or neutral Sephadex resins. The possibility of electrostatically induced migration to the anion exchange beads was not tested in the current study. The non-interaction of the hemocytes with the cation exchange beads suggested that either the hemocytes were repelled and/or their migratory activity was inhibited.

The contact between the DEAE resin and the walled stages and protoplast stage of E. egressa, the release of the test particles from neutralized DEAE beads and their failure to contact the CM resin suggested that these fungal stages are negatively charged. If charge repul-



sion is assumed to be the reason for the failure of the hemocytes of the hemlock looper to adhere to the protoplasts, then the hemocyte interaction with the walled stages may be indicative of a lower magnitude of negative charge on the walled stages. Charge repulsion could not be ruled out because it was not possible to measure distances between mobile protoplasts and freely floating hemocytes. Adhesion of the hemocytes to the glass surfaces may have prevented any detectable repulsion between the hemocytes and the more massive and, therefore, less easily repelled protoplasts. The less massive protoplasmic extensions of the protoplasts did not show signs of repulsion by sessile hemocytes. This may reflect, in part, the high momentum generated by the rapidly moving extensions and the active generation of mobility at the tips of the extensions.

Streams and Greenberg (1969) and Nappi and Streams (1969) proposed active suppression of hemocyte activity by parasitoid secretions. Because nylon fibers added to protoplast-hemocyte suspensions were actively encapsulated, the protoplasts were not believed to actively inhibit the hemocytes.

Vinson (1977) has proposed several passive strategies for parasitoid evasion of host-hemocyte responses. As



they relate to the protoplasts of E. egressa, these may include the acquisition of host hemolymph macromolecules by the surfaces of the protoplasts - thus, preventing the recognition of non-self particles by the hemocytes, the innate possession of chemically non-reactive surfaces, the presence of inhibitory substances on the protoplast surface or the evolution of molecular mimicry as considered by Damian (1964). These aspects will be dealt with in greater detail in section II. B. 2.



B. Choristoneura fumiferana.

1. Results.

(i) In vivo injections.

(a) Protoplasts of Entomophthora egressa.

The spruce budworm hemocytes were never observed adhering to the protoplasts of either isolate (Plate 8, Fig. 1). The protoplasts were, however, detected making active, short-lived contact with the five types of hemocytes in the spruce budworm (prohemocytes, plasmatocytes, granular cells, spherule cells and oenocytoids) (Plate 8, Fig. 2). In all protoplast-injected larvae E. egressa produced conidiophores 78h after injection. This occurred with larvae injected with either protoplast isolate. The control larvae developed normally and eventually pupated.

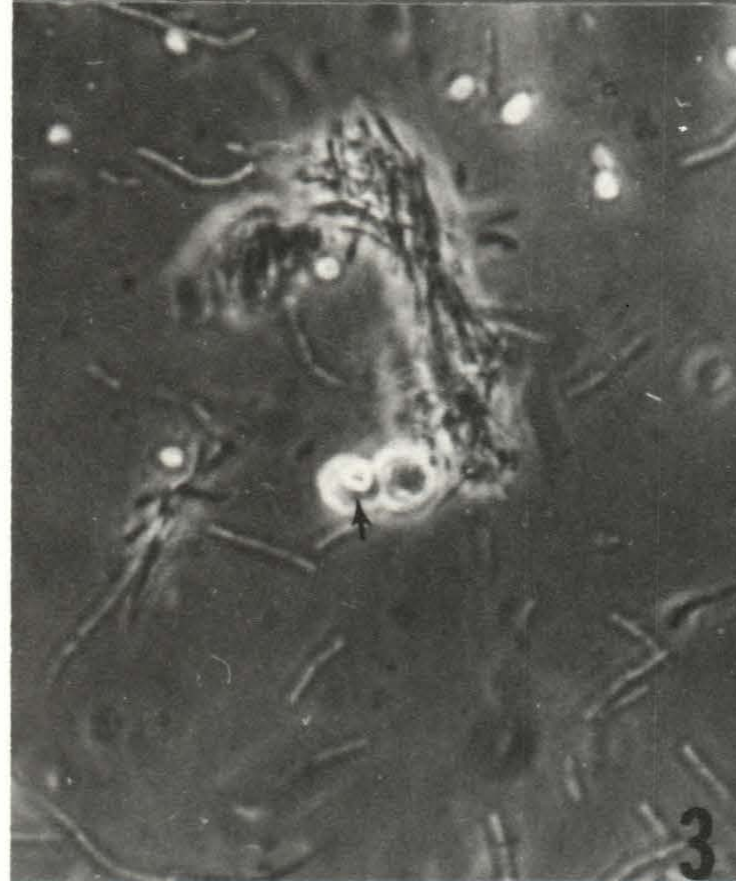
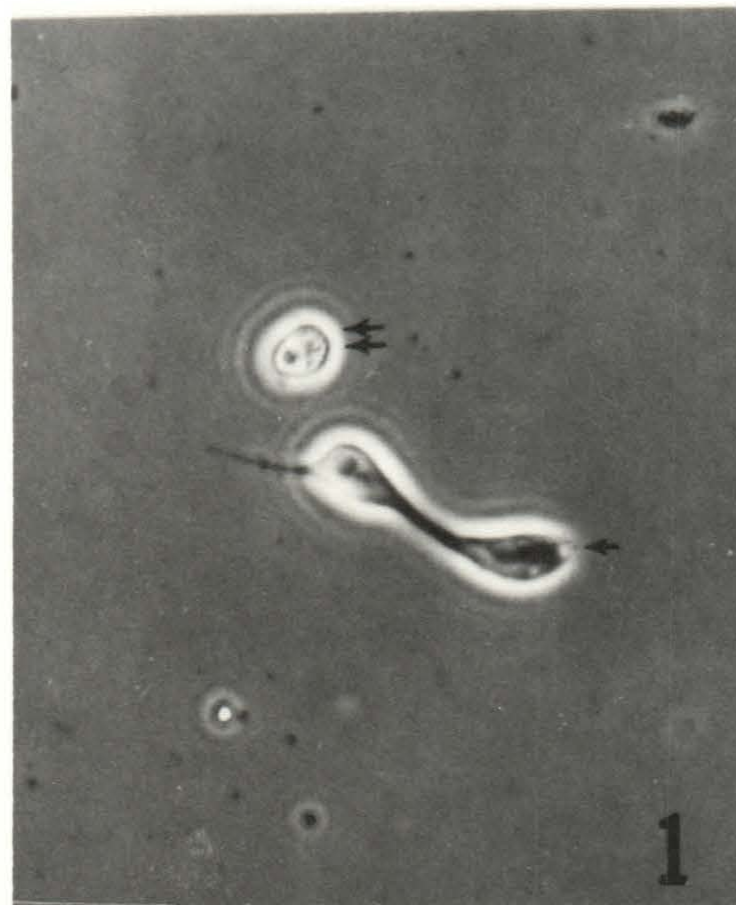
(b) Cells of Bacillus cereus and Escherichia coli. Both types of bacteria, although not totally cleared from the hemolymph, were predominantly located adhering to the granular cells. The bacteria were also located in vacuoles in these hemocytes (10% incidence) (Plate 8, Fig. 3). An average of  $3 \pm 1$  bacterial cells per granular cell were detected for either E. coli or B. cereus. Occasionally aggregates of granular cells



## Plate 8

- Fig. 1. Interaction between the protoplast stage of Entomophthora egressa (arrow) and the hemocytes of the spruce budworm (double arrows) in vivo. Phase contrast. X 1260.
- Fig. 2. Protoplast breaking contact with granular cell in vivo. Phase contrast. X 1260.
- Fig. 3. Bacillus cereus located in a vacuole (arrow) in a spruce budworm granular cell. In vivo. Phase contrast. X 1260.
- Fig. 4. Fungal protoplast (arrow) with protoplasmic extensions incubated in vitro with spruce budworm hemocytes. Phase contrast. MccT. X 1260.







were detected entrapping bacterial cells. The plasmatocytes also contained envacuolated bacterial cells (11% incidence). The plasmatocytes did not take part in the present phase of nodulation.

(c) Short-term effects of protoplasts and *Escherichia coli* on spruce budworm hemograms. Changes in THC reflected the nature of the test particles. The levels of THC declined below those of the non-injected control larvae (Fig. 1). The THC of the protoplast-injected larvae did not drop to the levels of the MGM or *E. coli*-injected insects. The THC recovery rate and the final level achieved also varied according to the test particles used. Larvae injected with *E. coli* did not show an increase in THC until 60min postinjection (Fig. 1) by which time the bacterial cells had been cleared from the hemolymph (Fig. 2). The MGM-injected larvae returned to the control levels 20min post-injection. Larvae injected with protoplasts achieved control THC values 60min post-injection. The recovery pattern differed from that of the MGM-injected larvae. The level of protoplasts remained constant throughout the 60min (Fig. 2). Because both *E. coli* cells and the protoplasts in the control media did not detectably increase in numbers (Fig. 2), it is believed that they did not multiply in vivo.

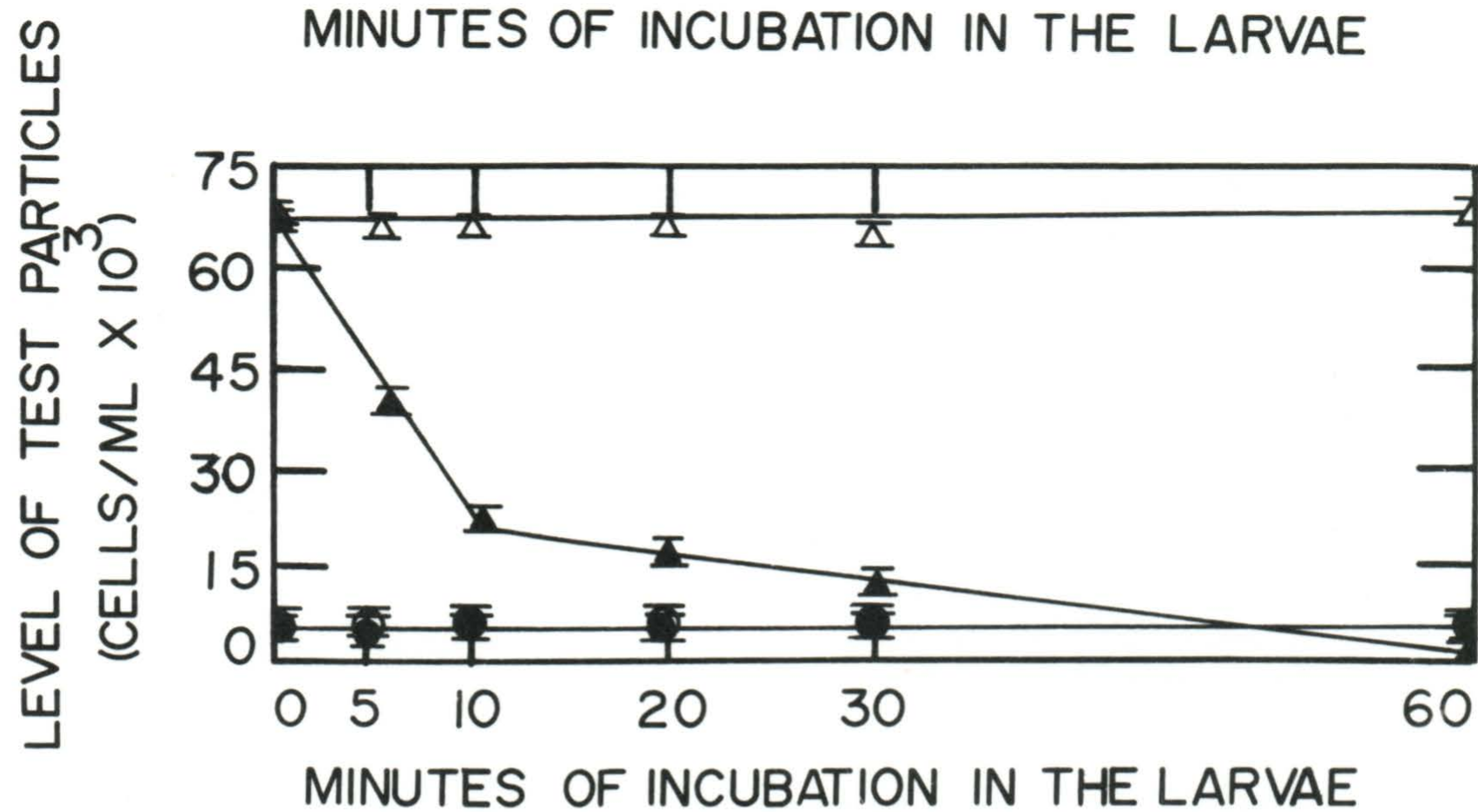
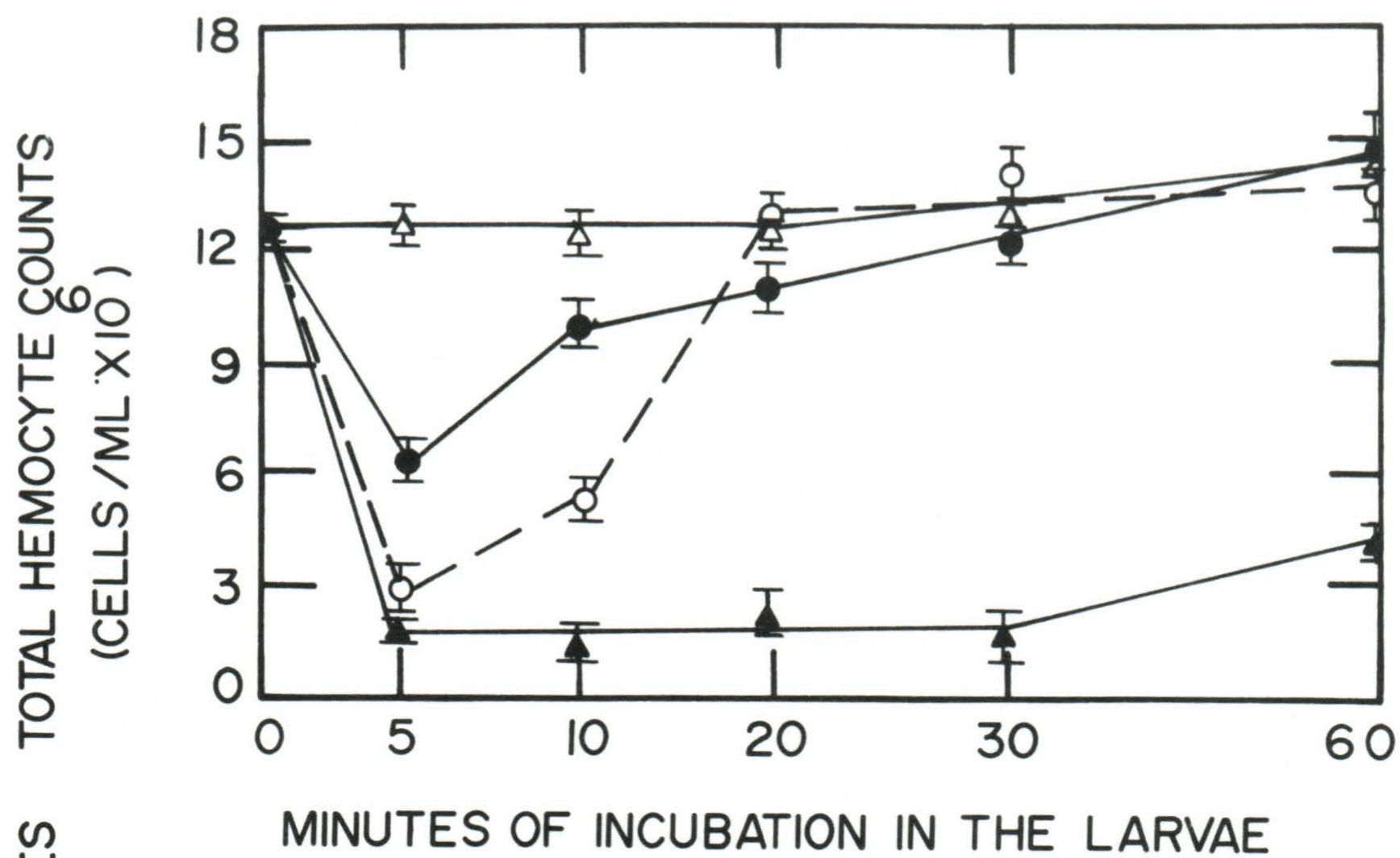


Fig. 1. Decline in total hemocyte counts of female sixth instar spruce budworm larvae injected with Escherichia coli (▲), protoplasts of Entomophthora egressa, isolate 521 (●), MGM (○) and non-injected larvae (Δ).

Fig. 2. Level of Escherichia coli cells in spruce budworm hemolymph in vivo (▲), in control medium (Δ) and protoplast levels of Entomophthora egressa in vivo (●) and in vitro in MGM (○).

All points represent mean values, the vertical lines represent standard errors.







(ii) In vitro incubation.(a) Protoplasts of Entomophthora

egressa. Initially both protoplast isolates in the control and hemolymph media were active, however, they became quiescent with time. Compared to the protoplasmic extensions (PE) of both isolates of protoplasts in the control medium, the PE of the cells in the hemolymph supplemented medium were more active, longer in length ( $19.9\mu\text{m} \pm 3.3\mu\text{m}$  (pooled) and  $35.6\mu\text{m} \pm 6.3\mu\text{m}$  (pooled) respectively;  $t=5.407$ ,  $P<0.001$ ) and more abundant ( $5.4\% \pm 0.9\%$  (pooled) and  $71.4\% \pm 0.4\%$  (pooled) respectively;  $t=164$ ,  $P<0.001$ ).

The hemocytes did not adhere to either protoplast isolate (Plate 8, Fig. 4); however, PE were often in brief contact with the granular cells. These filopodial-like extensions moved randomly until either making surface contact with a hemocyte or near a hemocyte after which they remained in the vicinity of the hemocyte for several seconds. The hemocytes readily adhered to fragments of nylon in the presence of the protoplasts.

(b) Interaction of the protoplasts

with granular cells of Tenebrio molitor. Within 5min of incubation, the PE usually increased from one per protoplast to 5 per protoplast for both isolates. The proto-



plasts became very pleomorphic. The PE adhered to only the granular cells. This induced the protoplasts to move away from the hemocytes in addition to causing rapid oscillation of the PE. During this time the number of PE increased to 8 to 10 per protoplast. Granules were commonly observed moving from the protoplasts towards the tips of the PE and into the medium (Plate 9, Fig. 5). Sometimes the PE broke free from the hemocytes; however, the protoplast usually left the PE behind in contact with the hemocytes (Plate 9, Fig. 6). The PE were not observed in the hemocyte controls (Plate 9, Fig. 7). Any contact by the protoplasts with other hemocyte types did not produce the described protoplast activity. Neither protoplast isolate was encapsulated. Protoplasts exposed to T. molitor serum possessed 5 PE per cell ( $SE=0.1$ ,  $n=50$ ).

(c) Hyphal bodies of *Entomophthora egressa*.

The spruce budworm hemocytes did not adhere to rod-shaped hyphal bodies (Plate 10, Fig. 8), spherical hyphal bodies (Plate 10, Fig. 9) or germinating spherical hyphal bodies (Plate 10, Fig. 10) of either isolate. The hemocytes readily adhered to nylon fibers added to these incubation mixtures (Plate 10, Fig. 11).

(d) Hyphae of *Entomophthora egressa*.

The granular cells adhered to the hyphae and hyphal tips



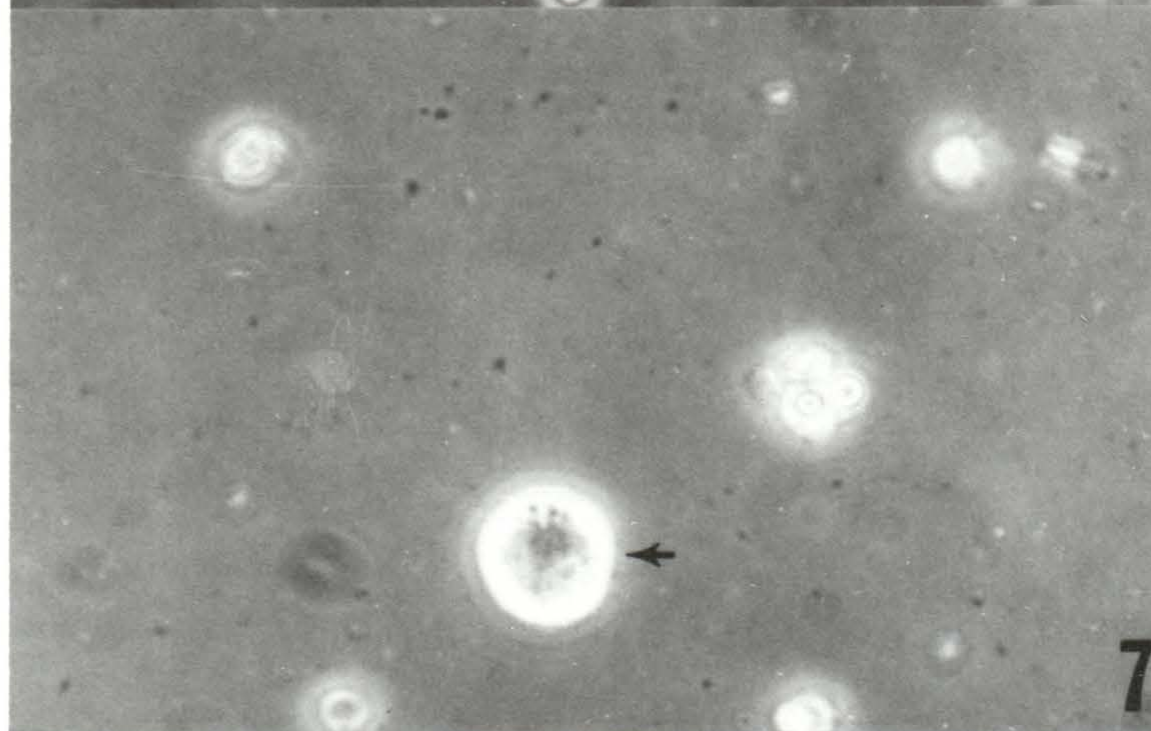
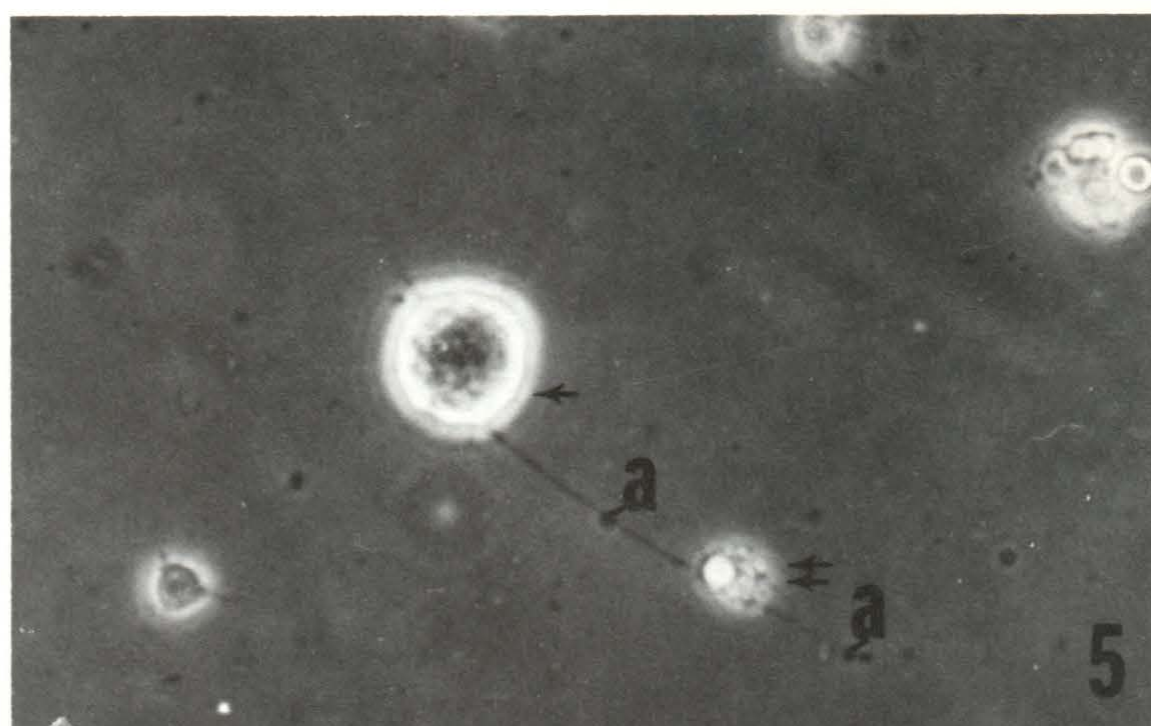
## Plate 9

Fig. 5. Protoplast (arrow) in contact with a granular cell of Tenebrio molitor by a protoplasmic extension (triple arrows) revealing granules ("a"). In vitro. Phase contrast. MccT. X 1260.

Fig. 6. Granular cell of Tenebrio molitor with protoplasmic extensions (arrows). In vitro. Phase contrast. MccT. X 1260.

Fig. 7. Granular cell (arrow) in hemocyte control without protoplasmic extensions. In vitro. Phase contrast. MccT. X 1260.



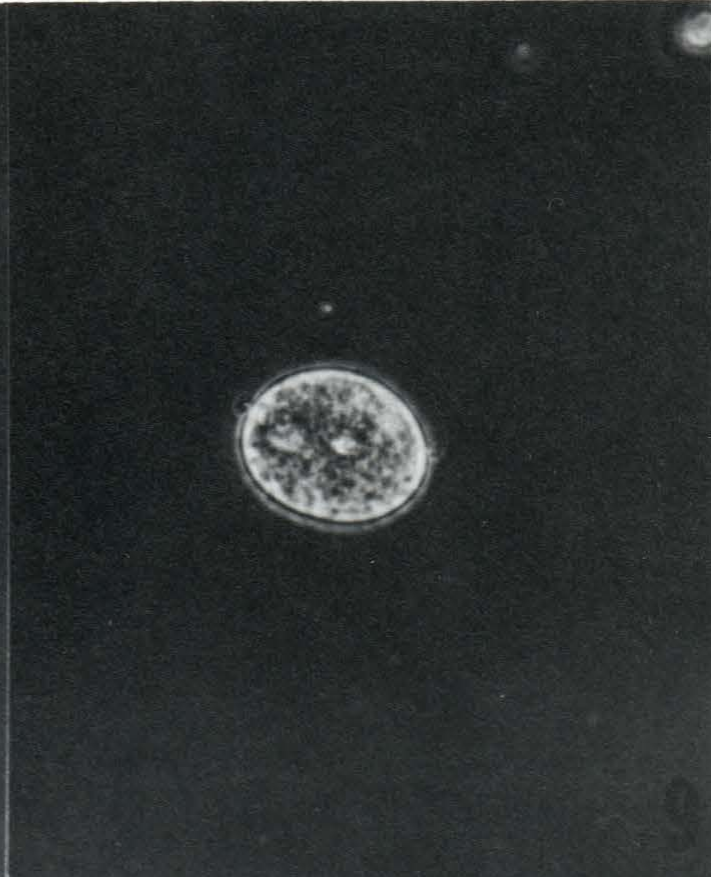




## Plate 10

- Fig. 8. Rod-shaped hyphal body of Entomophthora  
egressa incubated with spruce budworm hemo-  
cytes. In vitro. Phase contrast. MccT.  
X1100.
- Fig. 9. Spherical-hyphal body of Entomophthora  
egressa incubated with spruce budworm hemo-  
cytes. In vitro. Phase contrast. MccT.  
X1100.
- Fig. 10. Germinating spherical-hyphal body with  
surrounding hemocytes (arrow). In vitro.  
Phase contrast. MccT. X1100.
- Fig. 11. Granular cells (arrow) adhering to nylon  
fibers. In vitro. Phase contrast. MccT.  
X1100.







of both isolates in the six types of suspensions (Plate 11, Figs. 12 and 13). Hemocyte mobility was detected only when isolate 521 was incubated in spent and fresh MGM. In the spent medium cytoplasmic networks connected sections of hyphal fragments (Plate 11, Figs. 14 and 15). In fresh medium tracks were detected as the plasmatocytes moved away from the hemocyte aggregations (Plate 11, Figs. 16 and 17).

Although it was not possible to quantitate the number of hemocytes per hyphal fragment, it was possible to indirectly assess the extent of hemocyte adhesion by comparing the number of free hemocytes and the percentage of granular cells in each type of incubation medium (Table VII). There was no significant difference in the number of free hemocytes ( $F=0.292$ ,  $P>0.75$ ) or the level of granular cells ( $F=0.262$ ,  $P>0.6$ ) between the test media.

(e) Sephadex beads and spruce budworm hemocytes. Only the DEAE-Sephadex at pH4.2 attracted the hemocytes, especially the granular cells (Plate 12, Fig. 19) and the plasmatocytes (Plate 12, Fig. 20). No hemocytes adhered to the CM-Sephadex at pH4.2, 6.2 or 7.2.

(f) Influence of larval serum on sporangiospore-hemocyte contact. Based on the number of spores per granular cell and the percentage of granular



## Plate 11

- Fig. 12. Hyphae of Entomophthora egressa in fresh MGM encapsulated by spruce budworm granular cells. In vitro. MccT. X950.
- Fig. 13. Hyphal tip (arrow) of Entomophthora egressa engulfed by spruce budworm granular cells. Phase contrast. In vitro. MccT. X950.
- Fig. 14. Granulocyte-produced net-work (arrow) connecting hyphae of Entomophthora egressa. Phase contrast. In vitro. MccT. X1000.
- Fig. 15. Figure as described in Fig. 15. Nomarski. X1000.
- Fig. 16. Granulocyte (arrow) moving away from hemocytes aggregated about a hypha of Entomophthora egressa in fresh MGM. Note slime track (double arrows). Phase contrast. In vitro. MccT. X1100.
- Fig. 17. Figure as described in Fig. 16. Nomarski. X1000.



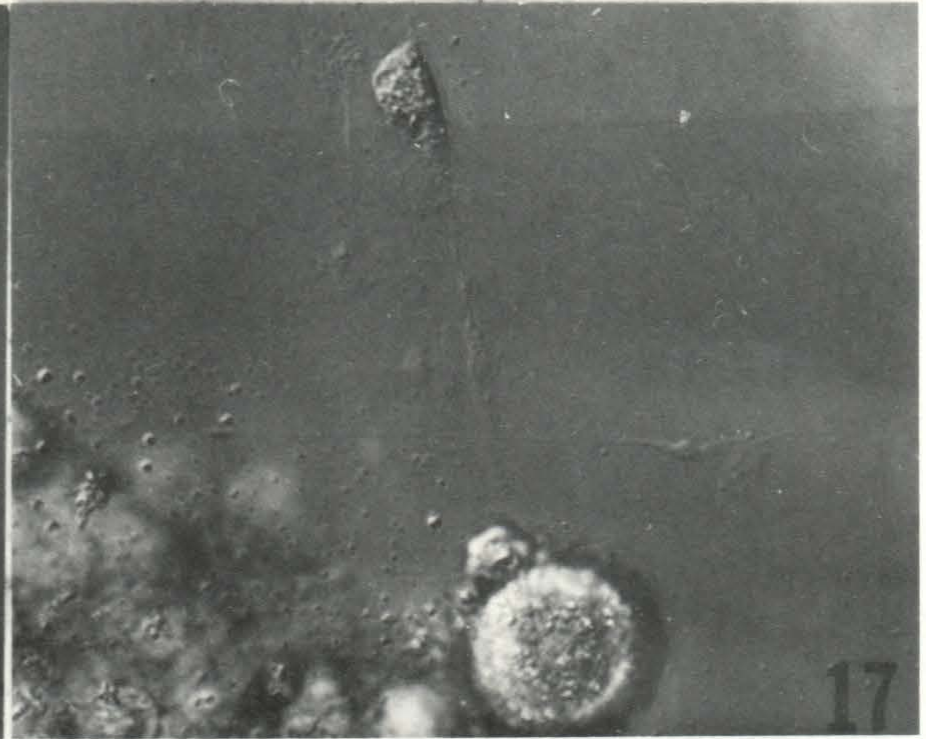
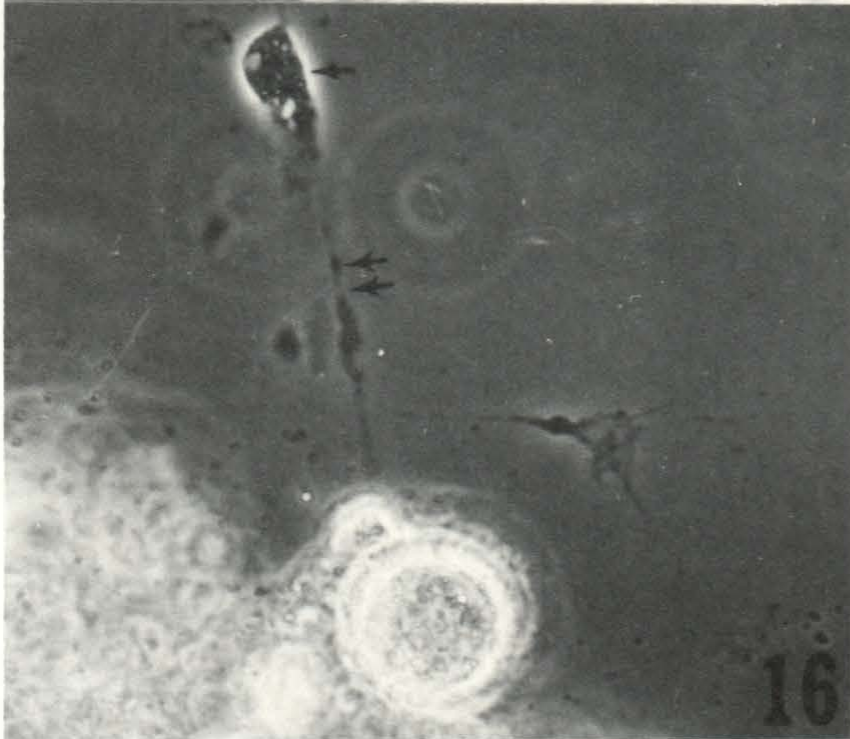
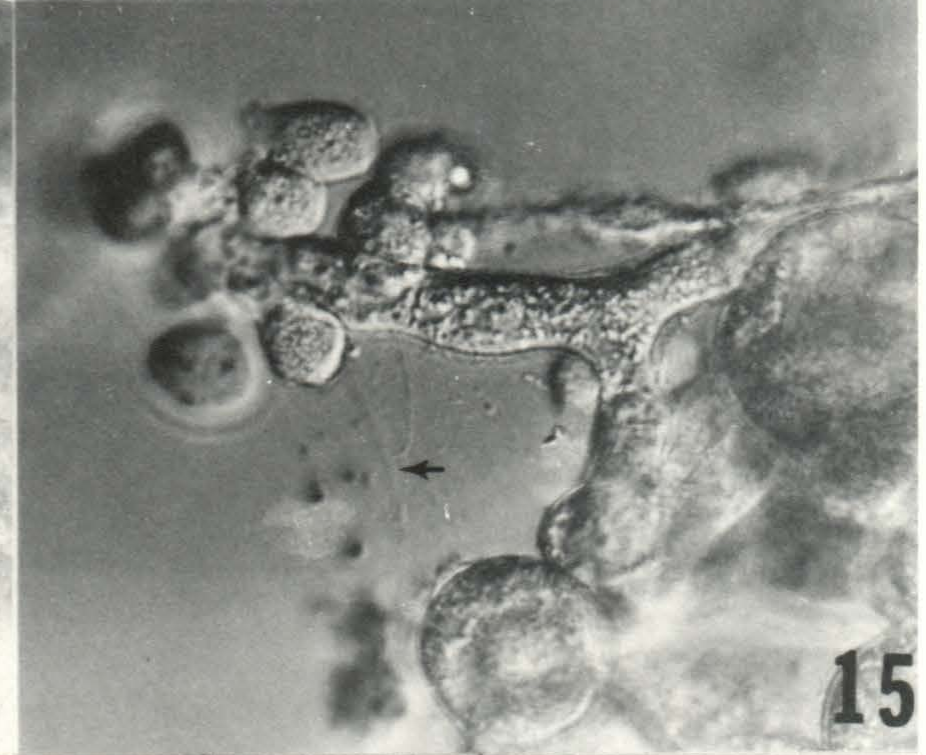
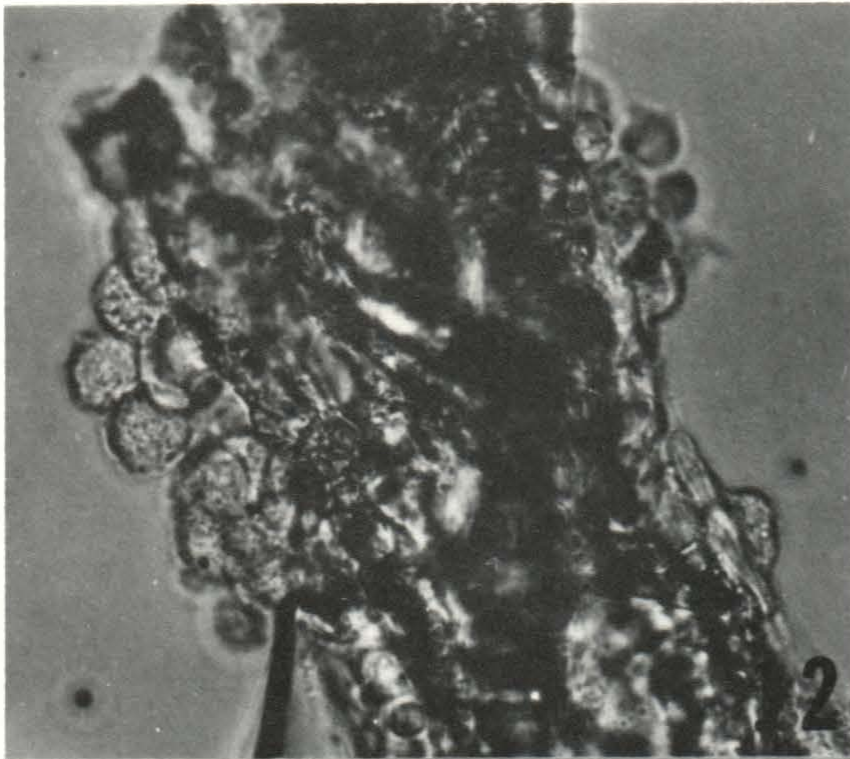




Table VII

The number of unattached hemocytes of the spruce budworm after incubation with hyphae of Entomophthora egressa isolates 458 and 521 in various forms of modified Grace's medium

Isolate used	Form of MGM <sup>a</sup>	Hemocyte level <sup>b</sup> ( $\pm$ SE, cells/ml $\times 10^3$ )	Granular <sup>b</sup> cells (%)
458	no previous growth	1.46 $\pm$ 0.52	62.1 $\pm$ 0.2
458	458 spent medium	1.52 $\pm$ 0.30	65.2 $\pm$ 0.9
458	521 spent medium	1.60 $\pm$ 0.21	60.7 $\pm$ 0.3
521	no previous growth	1.72 $\pm$ 0.31	59.9 $\pm$ 1.1
521	458 spent medium	1.71 $\pm$ 0.31	62.0 $\pm$ 0.7
521	521 spent medium	1.57 $\pm$ 0.53	67.9 $\pm$ 0.9

<sup>a</sup>Modified Grace's medium

<sup>b</sup>Sample size of 15

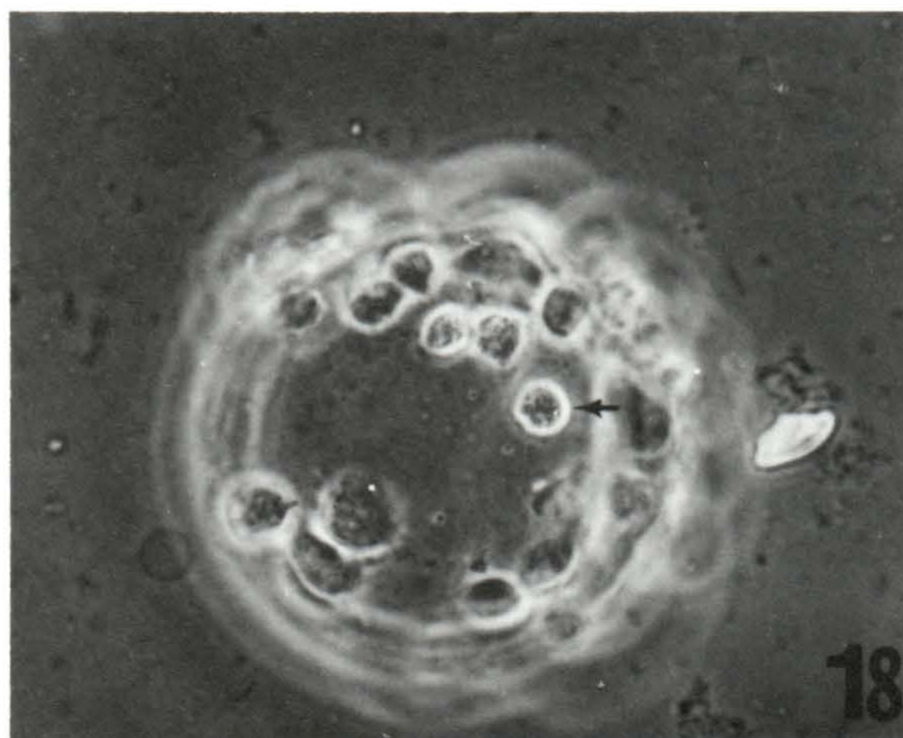


## Plate 12

Fig. 18. Granular cells (arrow) of spruce budworm larvae adhering to charged DEAE-Sephadex beads. Phase contrast. In vitro. MccT. X 250.

Fig. 19. Plasmatocytes (arrow) of spruce budworm larvae adhering to charged DEAE-Sephadex beads. Phase contrast. In vitro. MccT. X 250.







cells with spores, there was no evidence of serum influencing the interaction of the sporangiospores of A. repens with the spruce budworm hemocytes (Table VIII).

(g) Inhibition of hemocyte attachment to sporangiospores. The number of spores per granular cell in media without PTU were statistically equivalent for spores of A. repens and R. nigricans (Table IX;  $t=0.769$ ,  $P>0.55$ ). Similar results were observed when these spores were incubated in medium with PTU (Table IX;  $t=0.312$ ,  $P>0.85$ ). The presence of PTU decreased the degree of spore-hemocyte adhesion of A. repens ( $t=3.438$ ,  $P<0.001$ ) but not spore samples of R. nigricans ( $t=1.346$ ,  $P>0.05$ ).

The number of granular cells responding to spores of A. repens was greater than the number involved with spores of R. nigricans in the absence of PTU (Table IX;  $t=3.321$ ,  $P<0.05$ ). The presence of PTU did not affect the level of granular cells interacting with spores of R. nigricans (Table IX,  $t=0.157$ ,  $P>0.80$ ); however, PTU did effectively reduce the number of granular cells responding to A. repens (Table IX;  $t=4.235$ ,  $P<0.001$ ).

Compared to the hemolymph control samples, N-acetylglucosamine (331mM) enhanced the adhesion of the spores of A. repens to the larval hemocytes (Table X,  $t=5.213$ ,  $P<0.001$ ).



Table VIII

The influence of spruce budworm larval serum on the adhesion of the sporangiospores of Absidia repens to spruce budworm hemocytes

Suspension medium	Spores/ granular cell <sup>a</sup>	Granular cells with spores (%)
MGM	3.7 ± 0.2	62.3 ± 4.6
Serum	3.5 ± 0.1	69.7 ± 5.1
	n=10 <sup>b</sup>	n=10
	t=0.283	t=0.344
	(P>0.75)	(P>0.5)

<sup>a</sup>Values represent mean ± standard error

<sup>b</sup>Sample size



Table IX  
Interaction of spores of Absidia repens and Rhizopus  
nigricans with the hemocytes of the spruce budworm  
with and without the influence of phenylthiourea

Source of spores	Phenylthiourea	Spores/granular cell	Granular cells with spores (%)
<u>R. nigricans</u>	- <sup>a</sup>	2.8 $\pm$ 0.6 n=25 <sup>c</sup>	58.7 $\pm$ 4.4 n=25
<u>A. repens</u>	-	3.2 $\pm$ 0.4 n=25	79.6 $\pm$ 4.5 n=25
<u>R. nigricans</u>	+ <sup>b</sup>	2.0 $\pm$ 0.4 n=25	58.0 $\pm$ 0.6 n=25
<u>A. repens</u>	+	2.0 $\pm$ 0.4 n=25	58.0 $\pm$ 5.7 n=25

a- not present

b+ present (4 $\mu$ g/ml)

c sample size



Glucosamine yield results comparable to the control results ( $t=0.718$ ,  $P>0.4$ ). All other sugars and PTU reduced the adhesion of the spores ( $F=12.721$ ,  $P<0.001$ ). Sucrose, fucose, fructose, arabinose and galactose were among the more potent inhibitors.

During incubation the hemocytes were observed forming aggregates of varying sizes (Table X). With the exception of fucose, glucosamine, N-acetylglucosamine and PTU, the other reagents favoured substantial increases in the aggregation sizes over those of the controls ( $P<0.05$ ). Based on this, the aggregation frequency and granular cell measurements, there was no significant change in the total granular cell surface area available to the spores (Table X,  $F=0.329$ ,  $P>0.75$ ).



Table X  
Effects of selected carbohydrates and phenylthiourea  
on the adhesion of spores of Absidia repens to the hemocytes  
of female sixth instar larval spruce budworm

Compound		Spores/ granular cell (n=10) <sup>a</sup>	Granular cells/ aggregate (n=10)	Aggregation frequency (%) (n=10)	Total granular cell surface area ( $\mu\text{m}^2 \times 10^{-16}$ ) (n=10)
Trehalose	(346mM) <sup>b</sup>	2.7 $\pm$ 0.2	16.3 $\pm$ 0.7	10.2 $\pm$ 1.2	1.47 $\pm$ 0.07
Sucrose	(346mM)	1.9 $\pm$ 0.1	12.9 $\pm$ 0.6	26.2 $\pm$ 3.3	1.23 $\pm$ 0.06
Fucose	(359mM)	1.5 $\pm$ 0.1	11.0 $\pm$ 0.6	17.3 $\pm$ 3.1	1.37 $\pm$ 0.07
Arabinose	(370mM)	1.7 $\pm$ 0.1	17.2 $\pm$ 0.7	8.0 $\pm$ 1.0	1.51 $\pm$ 0.08
Fructose	(340mM)	1.8 $\pm$ 0.2	15.3 $\pm$ 0.7	33.6 $\pm$ 2.4	1.22 $\pm$ 0.06
Mannose	(340mM)	2.6 $\pm$ 0.0	13.4 $\pm$ 0.7	34.8 $\pm$ 2.8	1.29 $\pm$ 0.06
Galactose	(340mM)	1.8 $\pm$ 0.2	23.8 $\pm$ 0.8	29.4 $\pm$ 4.7	1.18 $\pm$ 0.05
Glucose	(340mM)	2.8 $\pm$ 0.3	18.2 $\pm$ 0.8	13.2 $\pm$ 1.0	1.43 $\pm$ 0.07
Glucosamine	(346mM)	4.0 $\pm$ 0.3	14.4 $\pm$ 1.7	10.3 $\pm$ 2.5	1.47 $\pm$ 0.07
N-Acetyl- glucosamine	(331mM)	5.5 $\pm$ 0.2	12.2 $\pm$ 0.7	22.4 $\pm$ 4.6	1.29 $\pm$ 0.05
Hemolymph <sup>c</sup>		4.2 $\pm$ 0.3	10.9 $\pm$ 0.6	12.0 $\pm$ 2.5	1.45 $\pm$ 0.08
Hemolymph <sup>d</sup>		2.1 $\pm$ 0.2	10.2 $\pm$ 0.7	11.9 $\pm$ 3.2	1.45 $\pm$ 0.07

<sup>a</sup>Sample size

<sup>b</sup>Carbohydrate concentration (mM)

<sup>c</sup>hemolymph diluted with homologous serum as a control

<sup>d</sup>as "b" plus 250 $\mu\text{g}$  of phenylthiourea/ml



2. Discussion. The observations that both isolates of E. egressa were able to develop in the spruce budworm and that, using in vivo and in vitro techniques, the hemocytes did not adhere to the protoplast stage of either isolate would suggest that the spruce budworm larvae are suitable for the fungal isolates. This does not mean, however, that the two isolates are equally adapted to the spruce budworm host. Whitcomb et al. (1974) have reviewed aspects of host-parasitoid adaptations. The absence of insect hemocytes adhering to fungal insect pathogens have been reported for Culicinomyces sp. in Dasyhelea sp. (Sweeney 1975) and for E. egressa isolate 458 in L. fiscellaria fiscellaria (section II. 1. i).

The spruce budworm granular cells, like those of L. fiscellaria fiscellaria, possessed phagocytic properties in addition to having a role in nodulation. The combined effect was the clearing of E. coli from the hemolymph within 60min. Phagocytic granular cells have been described by others (Yeager 1945; Takada and Kitano 1971; Whitten 1964; Stang-Voss 1970; and Takeda 1977).

The plasmatocytes, like those of the eastern hemlock looper (section II. A. 1. iii), had limited phagocytic activity. In both studies the plasmatocytes may reflect incomplete functional differentiation between plasmatocytes



and granular cells while morphological differentiation appeared to be complete at the light microscope level. Evidence for such transitions have been documented by Shrivastava and Richards (1965) and Landureau and Grellet (1975) and implicated by numerous electromicrographs (see Price and Ratcliffe 1974 and Raina 1976) and discussed by Arnold (1979).

In the present study the granular cells initiated nodulation. Wittig (1965, 1966) believed that granular cells with phagocytosed bacteria became adhesive for neighboring granular cells. Marchall (1966) proposed that bacteria adhered to the surfaces of the granular cells forming giant cells. Vey (1968), Vey et al. (1973), Vey and Vago (1969) and Vey and Farques (1977) proposed that nodules were formed by enveloping plasmatocytes. Gagen and Ratcliffe (1976), Ratcliffe and Gagen (1976, 1977) and Ratcliffe and Rowley (1979) proposed that nodulation was a biphasic process initiated by coagulation of hemolymph as a result of granule discharge from the granular cells induced by contact with the bacteria. The present results strongly support the proposal of Ratcliffe and his co-workers.

The granular cells of the spruce budworm rapidly initiated nodulation. This paralleled a decline in both



the THC and the number of E. coli in the hemolymph. Reports of such hemocytopenia are common (Whitcomb et al. 1974, Wittig 1965, 1966 and Gagen and Ratcliffe 1976). Ryan and Nicholas (1972), Hoffmann et al. (1974) and Gagen and Ratcliffe (1976) quantified the clearance rate of test particles from P. americana, L. migratoria and G. mellonella, and P. brassicae, respectively. The rates varied from 1-4h and may represent the inoculum level and species differences. As in the present study, Gagen and Ratcliffe (1976) reported that the initiation of nodulation was rapid. In agreement with Ratcliffe et al. (1976), the granular cells of the spruce budworm larvae are responsible for the identification of foreignness.

The rise in THC values following the initial decrease in larvae injected with E. coli, protoplasts or MGM may reflect sedentary hemocytes entering the circulation. Gagen and Ratcliffe (1976) implied that because nodules still existed in P. brassicae when the THC levels increased the hemocytes came from possible hemocytopoietic organs. In the present study the increase in THC in spruce budworm containing nodules may also reflect the presence of hemopoietic organs. The slow rise in THC in larvae with E. coli may be indicative of an overloaded hemocyte response in which the bacteria were so numerous that as granular cells entered the hemolymph they would be removed by



nodulation. The THC level did not increase until all detectable E. coli cells had been removed.

The failure of the THC of protoplast-injected larvae to decline to the level of the MGM-injected larvae or to recover in parallel fashion suggested that the hemocytes recognized the protoplasts or its metabolites as non-self agents. A putative chemotactic role for G. mellonella plasmatocytes against conidia of A. flavus has been documented by Vey et al. (1968). Gagen and Ratcliffe (1976), Ratcliffe and Gagen (1976) and Ratcliffe and Rowley (1979) have documented chemotaxis by G. mellonella plasmatocytes toward Bacillus cereus coated with granulocyte discharge. They proposed that the granular cells induced hemocyte attraction.

The protoplast activity induced by the hemolymph of T. molitor and during hemocyte contact appeared to confer the advantage of avoiding hemocyte adhesion in vitro. The significance of this in vivo remains to be determined, but it may allow the pathogen to have a broad host range. The discharge of protoplast granules in contact with hemocytes may represent an attempt to confuse the hemocytes in a fashion similar to that proposed by Powell (1976) for C. punctatus in A. quadrimaculatus.



The increase in PE numbers on protoplasts in contact with T. molitor hemocytes compared to protoplasts in T. molitor serum suggests that the protoplasts recognize unfavourable cells. The enhanced PE level for protoplasts in serum over those in MGM may reflect the enriched nutrient condition of the serum and/or humoral antiprotoplast activity.

The hyphal bodies, regardless of the developmental stage, did not react with any of the spruce budworm hemocyte types. The granular cells, however, readily adhered to the hyphae of both isolates of E. egressa. The spherule cells of L. fiscellaria fiscellaria adhered to both the hyphal bodies and hyphae of E. egressa isolate 458. The nature of the test particles and the host species influenced the type of hemocyte response.

The results using DEAE-Sephadex beads, while establishing the existence of negatively-charged hemocytes, do not clarify the possible electrostatic interaction between the hemocytes and protoplasts. These aspects have been considered in section II. A. 2.

Neither protoplast isolate suppressed the adhesion of hemocytes to either nylon fibers or hyphal fragments of either isolate of E. egressa. This fact, in conjunction



with the in vivo recognition of the protoplasts by the hemocytes, strongly suggests that the failure of the hemocytes to adhere to either protoplast isolate is not due to active hemocyte suppression but possibly due to some aspect of molecular mimicry or suppression of hemocyte activity at the protoplast surface. Nappi (1975) has reviewed the mechanisms by which successful parasitoids suppress or resist host hemocytes including molecular mimicry and the secretion of hemocyte inhibitory substances.

The equal levels of unattached granular cells in the present study reveals the absence of adhesion selectivity by the granular cells.

The induction of different types of hemocyte mobility are unexplainable at this time. Hemocyte induced mobility has also been observed for hemocytes of L. fiscellaria fiscellaria contacting R. nigricans hyphae.

The failure of larval serum to enhance the adhesion of A. repens spores to spruce budworm granular cells argues for the absence of serum opsonins. Phagocytosis was also not enhanced. The absence of humoral-mediated non-self recognition is common among insects (Ratcliffe and Rowley 1979). Serum-independent attachment by insect hemocytes is probably mediated, at least partially, by interactions



with cell surface receptors.

The percentage of spruce budworm granular cells with spores of R. nigricans and A. repens suggests specificity in response. This proposal of specificity is further enhanced by the selective inhibition of the spores in the presence of PTU. That PTU inhibits hemocyte activity has been reported by Brewer and Vinson (1971), Nappi (1973) and Beresky and Hall (1977). The mechanism of inhibition is not known. In the present case, it may be that specific receptors for the 2 fungi exist on the hemocytes and differ in susceptibility to the action of PTU.

The adhesion of the granular cells to hyphae of E. egressa implies that chitin or its monomer, N-acetylglucosamine (GlcNAc) are involved in triggering the hemocyte response. Chitin is a common cell wall component of the Entomophthorales (Hoddinott and Olsen 1972). N-acetylglucosamine strongly enhanced the adhesiveness of the spruce budworm granular cells to the fungal spores and glucosamine (GlcA) yielded results comparable to the control levels. All other test sugars reduced hemocyte adhesion. It is plausible, therefore, that the cell wall of the hyphae interacting with the appropriate hemocyte receptors induced adhesiveness. Unestam and Beskow (1977) reported that the cell walls of Saccharomyces cerevisiae Hansen, Polyporus annosus



(Fries) Cooke and A. astaci triggered hemocyte activity in A. astacus. Chadwick and Aston (1976a) reported that carbohydrates on the walls of Pseudomonas aeruginosa induced immune responses in G. mellonella. The failure of the granulocytes of C. fumiferana to adhere to the hyphal bodies, in view of the above, suggests that the hyphal body cell walls may have possessed only inhibitory carbohydrates and/or lacked stimulatory carbohydrates.

Ratcliffe (1975), Ratcliffe and Gagen (1976, 1977) and Schmidt and Ratcliffe (1977) have proposed that non-self agents either by inducing stress on the hemocyte plasma membrane or by other means (= receptor triggered?) induce the granular cells to discharge their granules forming an adhesive layer around the hemocytes which favoured the adhesion of these hemocytes to non-self particles. In the present context the stimulatory effects of GlcNAc and the inhibitory effects of the other sugars could be best explained using the model proposed by Ratcliffe and his co workers.

The results were not believed to be attributable to osmotic stress because all the test solutions were at 350 mOsM/Kg (the value of spruce budworm hemolymph, see section IX. A. 1.) and the granular cells did not reveal evidence of osmotic imbalance.



The increasing levels of granular cells with spruce budworm larval development (section I. A. 2.) and the differences between the sexes may be indicative of differences in cellular defenses. Ratcliffe (1979) has speculated on sex as a factor influencing the hemocytic encapsulation reaction.



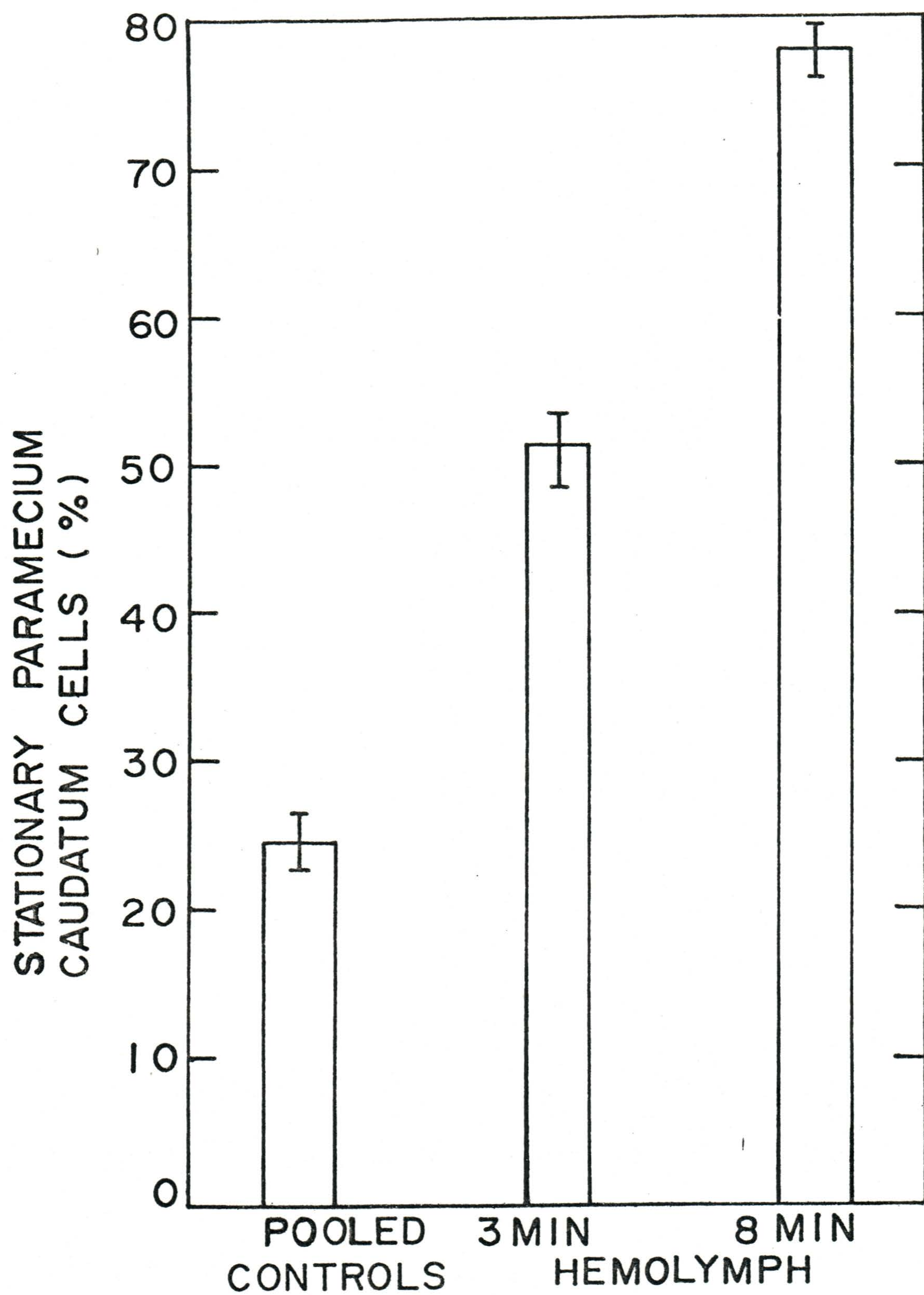
III. Protozoan lysins in the larval eastern spruce  
budworm hemolymph.

A. Results. During a study of how the hemocytes responded to moving foreign protozoa, it was noticed that the hemocytes never adhered to either P. caudatum or E. gracilis even when the protozoa collided with the hemocytes. The same behavioural patterns were detected for the protozoa in the presence or absence of PTU in the hemolymph regardless of the incubation temperature. Within the first 90sec of exposure to hemolymph, cells of P. caudatum and E. gracilis were seen to be much more mobile than those in the control media. This activity decreased within 3-5min and continued to decrease until 20-30min of incubation by which time partial lysis was evident. This decrease in activity was most pronounced for P. caudatum (Fig. 3). Ciliary activity continued even in stationary cells of P. caudatum. Trichocyst discharge, a sign of stress, was detected only in the samples of P. caudatum containing hemolymph and not in the control samples. Although several techniques were tested, it was not possible to quantitatively assess the effects of hemolymph on E. gracilis. Partial lysis of P. caudatum and E. gracilis was detected by the appearance of discrete holes with small blebs of extruding cytoplasm. In P. caudatum, ciliary activity continued as the orifice grew



Fig. 3. Stationary Paramecium caudatum cells incubated in modified Grace's medium and in larval eastern spruce budworm hemolymph. Vertical bars represent standard errors.







NUMBER OF SURVIVING  
PARAMECIUM CAUDATUM (CELLS/ML X 10<sup>3</sup>)

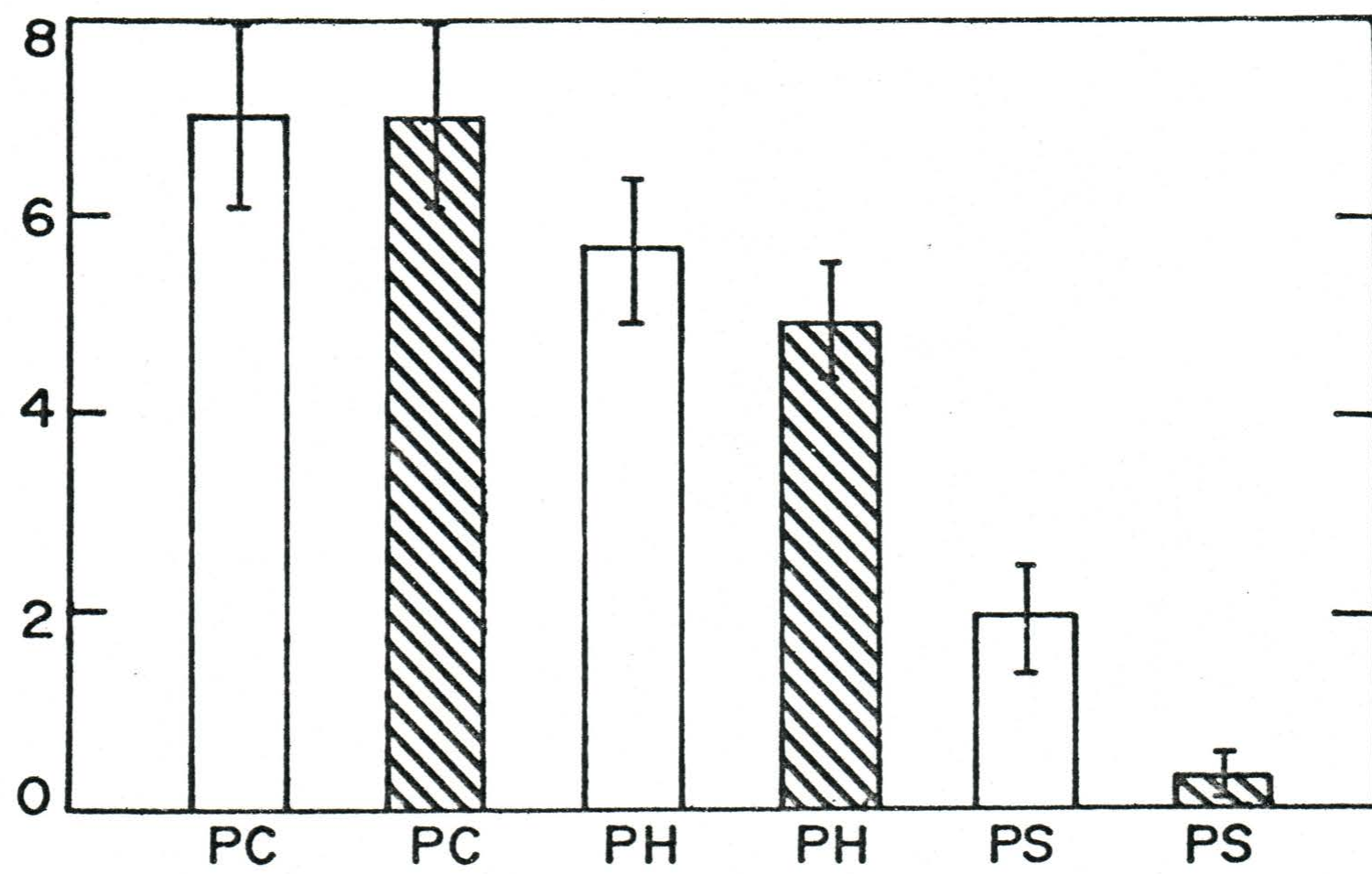




Table XII  
Results of incubating hemocytes of sixth instar  
larvae of Choristoneura fumiferana with Paramecium  
caudatum in modified Grace's insect tissue culture medium<sup>a</sup>

Type of sample	Total hemocyte counts (cells/ml $\times 10^6$ )	Differential hemocyte counts (%) <sup>b</sup>		
		Granular cells	Spherule cells	Unclassified hemocytes
Hemocyte control	$4.82 \pm 0.23^b$	$77.1 \pm 2.5$	$16.6 \pm 4.4$	$6.8 \pm 2.8$
Hemocyte plus <u>Paramecium</u> <u>caudatum</u>	$3.32 \pm 0.20$ ( $t=2.306$ , $P<0.05$ )	$77.7 \pm 3.7$ ( $t=0.604$ , $P>0.5$ )	$18.5 \pm 4.2$ ( $t=0.128$ , $P>0.9$ )	$5.4 \pm 3.0$ ( $t=0.139$ , $P>0.8$ )

<sup>a</sup>0 min incubation at 20°C



<sup>b</sup>mean  $\pm$  standard error, n=6

(Note: Only sixth instar female larvae used)

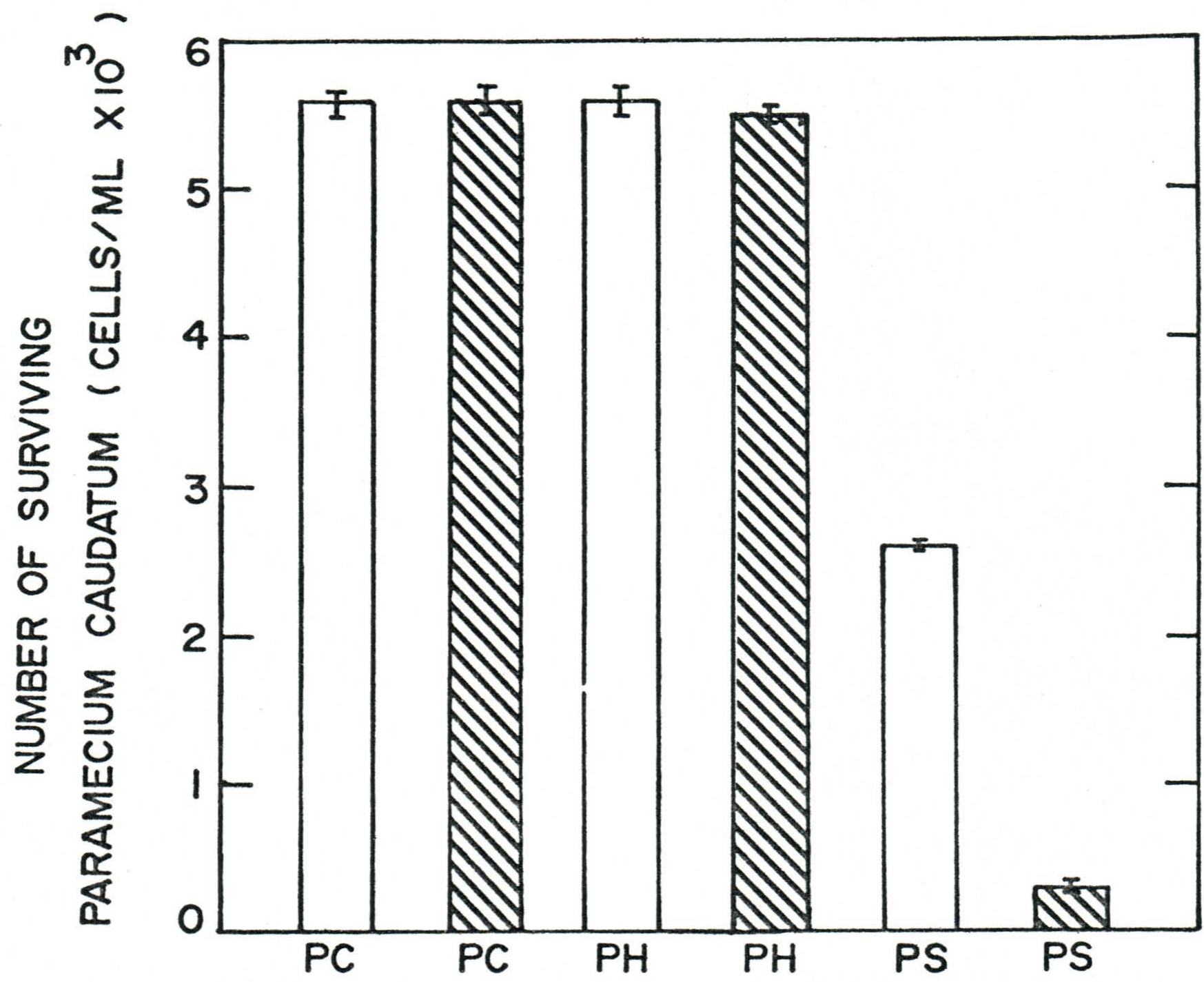


$1.3 \times 10^6 \pm 0.1 \times 10^6$  cells/ml;  $t=31.844$ ,  $P<0.001$ ). Heating for 5min produced results comparable to those for serum heated for 30min. Dialysed serum was as lytic as control serum.

The addition of PTU to serum or hemocyte preparations did not change the lytic pattern previously described (Fig. 5).

Fig. 5. Number of surviving Paramecium caudatum cells incubated with modified Grace's medium (MGM), hemocytes and serum in MGM in the presence of phenylthiourea for 30 min. Paramecium caudatum cells in MGM, controls (pooled) (PC), hemocytes plus P. caudatum cells (PH), serum plus P. caudatum cells (PS). Total number of P. caudatum cells . Number of intact cells .





B. Discussion. The evidence suggested that the lytic ability of the hemolymph was due to a heat-labile, non-enzymatic protein(s) in the serum fraction. The serum appeared to influence the hemocyte response during protozoan lysis. It is not known if the hemocyte response is to lysin-damaged protozoa or a direct interaction with some serum factor. Induction of antibacterial factors by plasma-hemocyte interaction has been reported in H. americanus (Stewart and Zwicker 1972). Weiser (1969) reported the presence of lysins in Baetis pumilus MacDunnough active against the microsporidian Nosema baetis. Lytic elements against protozoa have been detected in several non-insect invertebrate species (Bang 1966, Feng and Stauber 1968). A lysin against Pseudomonas aeruginosa (Schroeter) Migula has been detected in Oncopeltus fasciatus (Dallas) (Gingrich 1964). A proteinaceous component in the hemolymph of P. americana has been found to inactivate Tetrahymena pyriformis (Ehrenbert) (Seaman and Roberts 1968).

The absence of hemocyte-protozoan adhesion is unusual. Tobie (1968), Zeledon and Monge (1966), Weiser (1969) and Brooks (1970) reported that many parasitic protozoa not normally present in a given insect host were actively engulfed by insect plasmatocytes or hemocytes in general.

#### IV. Comparative physiology of two isolates of Entomophthora



egressa in selected stages of development.

Because of the absence of adherence of spruce budworm granular cells to the protoplasts of either isolate in vivo and in vitro, it was decided to compare the basic physiology of the isolates to assess the possibility of differences in isolate adaptation to a given host.

A. Results.

1. Growth on coagulated egg yolk medium. The two isolates revealed substantial differences in colonial morphology. Isolate 458 (I458) did not produce as thick a mycelial mat as isolate 521 (I521). In addition, the mycelial mat of the former (Plate 13, Fig. 1) was not as convoluted as that of the latter (Plate 13, Fig. 2). Isolate 458 generally grew submerged beneath the surface of the medium with irregular tufts of dry mycelium on the surface. Isolate 521 grew entirely on the surface as a glistening mat. Both isolates were cream white in color and liquified the solid medium directly beneath the fungal mats.

2. Growth in MGM.

(i) Growth and morphogenesis in MGM. The two isolates based on statical analyses of growth rates and cell yields grew at different rates and reached different

## Plate 13

Fig. 1. Entomophthora egressa, isolate 458, grown  
on coagulated egg yolk medium. X 4.

Fig. 2. Entomophthora egressa, isolate 521, grown  
on coagulated egg yolk medium. X 4.







maximum protoplast levels (Fig. 6). The population doubling time for I458, 6.2h during the early exponential growth phase, was less than that of I521, 7.2h ( $t=2.533$ ,  $P<0.05$ ). The growth rate of I458 declined after 48h of growth (population doubling time = 16.8h). Maximum protoplast levels occurred at 48h and 72h for I521 and I458, respectively (Fig. 6).

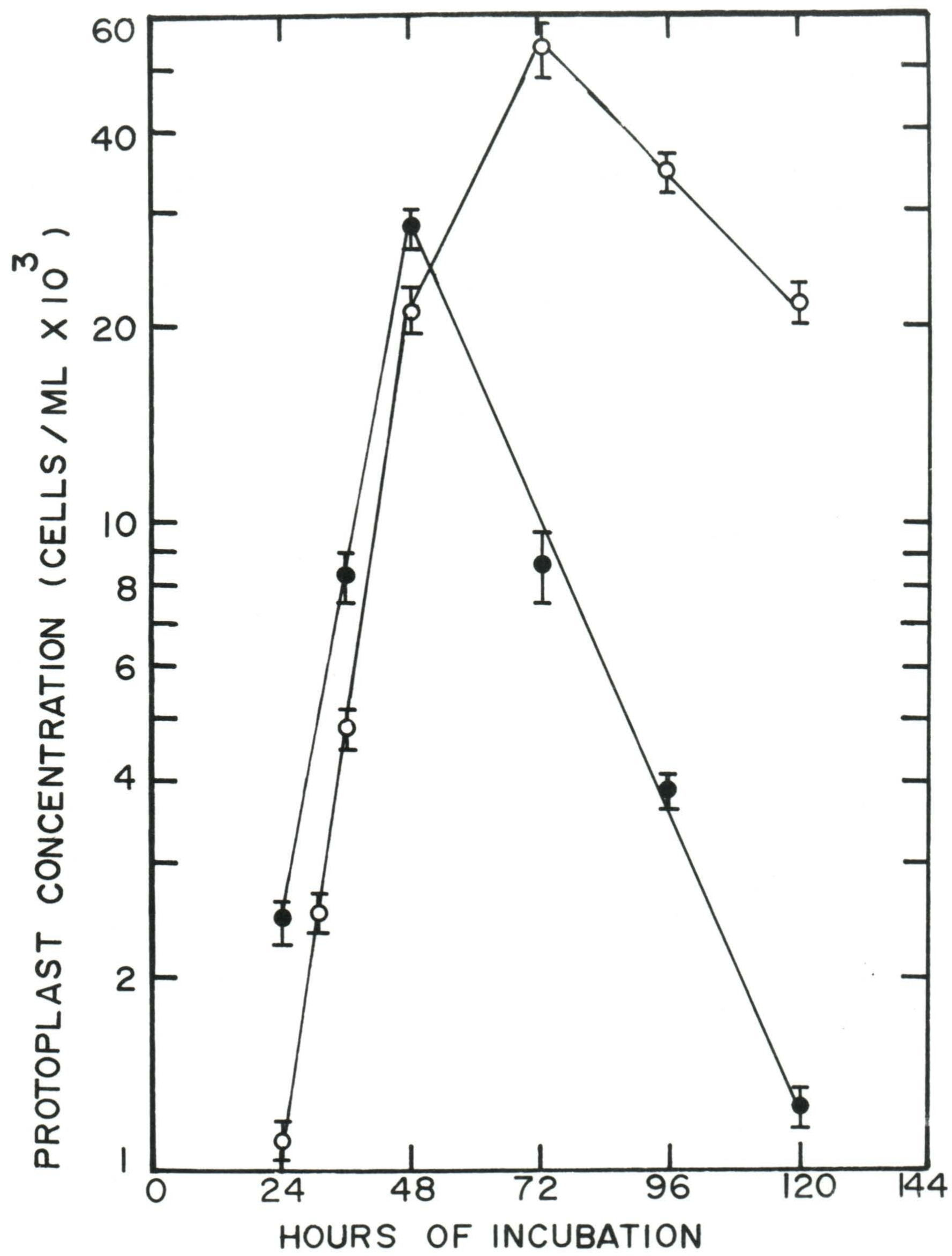
The morphogenetic sequences of protoplast regeneration for I458 paralleled the patterns of fusion-sphere regeneration reported by Dunphy and Nolan (1977a); whereas, the sequence of I521 resulted in the formation of spherical hyphal bodies described by Dunphy and Nolan (1977b).

(ii) Changes in MGM components during fungal development.

(a) pH and osmolality changes. The pH of MGM decreased from  $6.15 \pm 0.05$  at 24h to  $5.95 \pm 0.02$  by 48h for I521 (Fig. 7). A similar decrease was detected for I458 by 72h. During cell wall regeneration the pH values returned to control levels for both isolates. By 120h when 98% of the spherical hyphal bodies of I521 had settled out of suspension during aggregation and 96% of the fusion spheres of I458 had completed wall thickening, the pH of the medium for both isolates had declined to  $5.75 \pm 0.12$  and



Fig. 6. Growth of protoplasts of Entomophthora  
egressa, isolate 458 (O) and isolate  
521 (●) in modified Grace's insect tissue  
culture medium.





by 169h, the pH values were restored to the initial levels (Fig. 7).


The osmolality of the media remained essentially constant throughout most of the study (Fig. 7). The osmolality of MGM increased in cultures of I458 prior to maximum protoplast levels ( $t=7.934$ ,  $P<0.001$ ) and declined significantly by 72h ( $t=8.485$ ,  $P<0.001$ ). With the advent of fusion sphere-initials the value returned to the initial value. Unlike the changes in pH levels, I521 caused a slower different pattern of change in medium osmolality. The value increased substantially at 72h to 410mOsm/Kg ( $t=7.119$ ,  $P<0.001$ ) during the development of spherical hyphal bodies (Shb) and returned to the constant level of 375 10mOsm/Kg thereafter.


(b) Changes in NPC levels. The level of total identified NPC of MGM did not decline during protoplast growth of I458 but did decline by approximately 16% during the fusion sphere-initials stage at 72h to 49.8 mM  $\pm$  1.2mM (Fig. 8). This level was constant throughout the last residual protoplast growth to 120h and the heterogeneous stages of fusion sphere development until 144h during which time the level increased by 10% (Fig. 8). After 144h the total NPC level declined by 10%. For I521 cultures the NPC values remained constant throughout protoplast growth


Fig. 7.


Changes in the pH and osmolality levels  
of MGM during the development of Entomoph-  
thora egressa isolate 458 (0) and isolate  
521 (●)

Protoplast stage - 

Spherical hyphal body stage - 

Fusion sphere initial stage - 

Fusion sphere stage - 

Germinating spherical hyphal body - 

Hyphal stage - 

Hyphal lysis - 



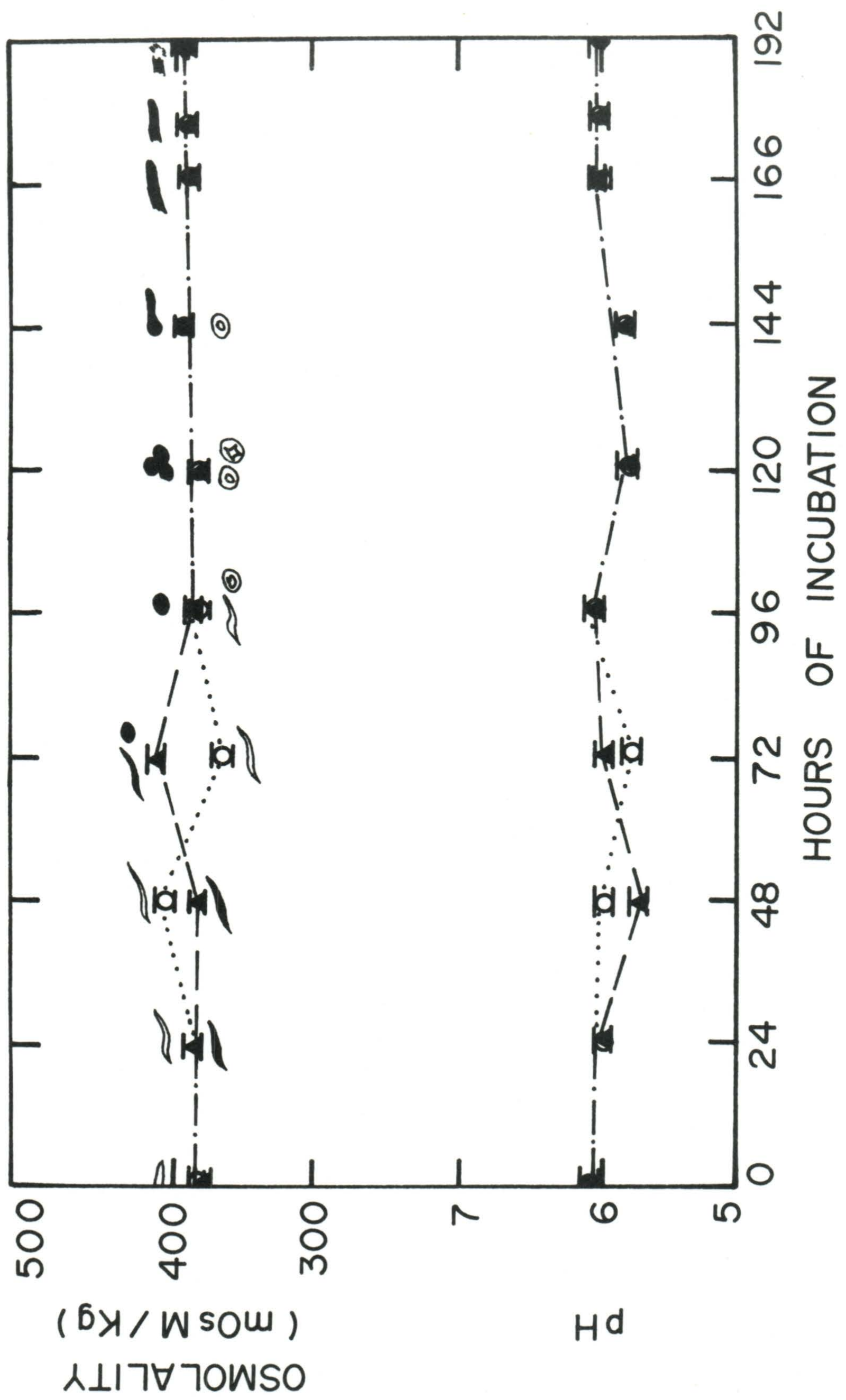
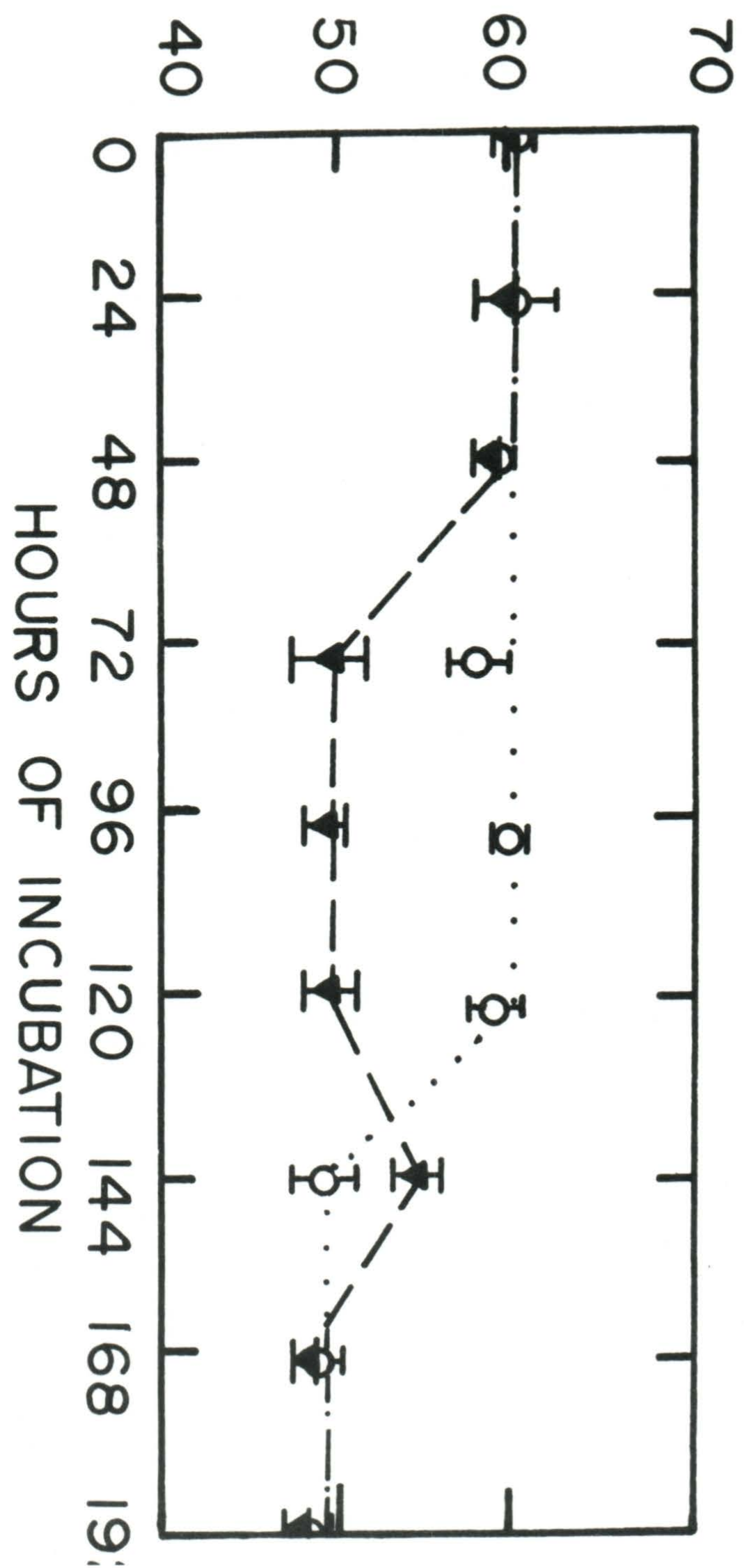


Fig. 8. Changes in the total ninhydrin-positive compounds of MGM during the development of isolate 458 ( $\Delta$ ) and isolate 521 (0) of Entomophthora egressa.



TOTAL IDENTIFIED  
NINHYDRIN-POSITIVE COMPOUNDS (mM)



(0-72h), Shb formation (72-96h) and Shb aggregation (96-120h). The level declined by 17% to 50mM prior to the onset of hyphal body germination by the majority of the spherical hyphal bodies (Fig. 8). The level remained constant thereafter (Fig. 8).

During the growth of the protoplasts of I458 and the initiation of fusion spheres (96-120h), the levels of L-aspartic acid, L-glutamic acid, L-lysine, L-histidine, L-tyrosine, L-leucine, L-valine, L-alanine, L-threonine, L-glutamine, glycine, and B-alanine decreased (Table XIII). During early fusion sphere activity (144h), these NPC's increased in magnitude by differing percentages accounting for the increase in total NPC (Fig. 8) and declined thereafter (Table XIII). The levels of DL-serine declined continuously throughout the study. The isolate did not appear to exhibit selective preferences for NPC on the basis of acidic, basic, polar or nonpolar properties. The levels of NPC in MGM during the development of I521 showed greater variability (Table XIV) than did those of I458, thus few significant changes in the levels of amino acids were seen. During the germination of spherical hyphal bodies the level of L-leucine declined by 20% to  $0.38\text{mM} \pm 0.02\text{mM}$  and remained at this level during later stages of fungal development. L-aspartic acid also declined during germination (Table XIV). Reduced levels of L-methionine and L-isoleucine occurred



Table XIII  
Changes in the levels of ninhydrin-positive compounds (mM) in modified Grace's insect tissue culture medium by the growth and development of several stages of *Entomophthora egressa* isolate 458

NPC <sup>a</sup>	Time (h) of incubation (h)								
	0	24	48	72	96	120	144	169	193
<u>Acidic</u>									
L-Aspartic acid	2.782 ± 0.056	2.594 ± 0.040	2.485 ± 0.180	2.040 ± 0.177	2.064 ± 0.177	1.225 ± 0.019	1.829 ± 0.218	1.847 ± 0.138	1.868 ± 0.034
L-Glutamic acid	4.009 ± 0.093	3.883 ± 0.068	3.686 ± 0.231	3.142 ± 0.249	3.183 ± 0.131	2.996 ± 0.168	3.556 ± 0.164	2.755 ± 0.137	3.007 ± 0.056
<u>Basic</u>									
L-Lysine	3.344 ± 0.071	3.376 ± 0.040	3.132 ± 0.305	2.735 ± 0.245	2.713 ± 0.255	2.590 ± 0.150	2.895 ± 0.112	2.508 ± 0.230	2.696 ± 0.029
L-Histidine	12.873 ± 0.022	12.194 ± 0.169	12.248 ± 0.277	10.648 ± 0.634	10.640 ± 0.622	10.496 ± 0.418	11.906 ± 0.334	10.613 ± 0.386	11.355 ± 0.193
L-Arginine	2.386 ± 0.166	2.678 ± 0.025	2.477 ± 0.242	2.471 ± 0.333	2.141 ± 0.200	2.044 ± 0.158	2.359 ± 0.186	1.949 ± 0.148	2.431 ± 0.295
<u>Nonpolar R groups</u>									
L-Tryptophan	0.495 ± 0.010	0.519 ± 0.008	0.460 ± 0.047	0.428 ± 0.024	0.400 ± 0.039	0.409 ± 0.043	0.456 ± 0.039	0.352 ± 0.030	0.350 ± 0.038
L-Phenylalanine	0.904 ± 0.019	0.897 ± 0.011	0.836 ± 0.075	0.716 ± 0.062	0.713 ± 0.046	0.665 ± 0.048	0.768 ± 0.039	0.601 ± 0.046	0.656 ± 0.019
L-Tyrosine	0.296 ± 0.015	0.314 ± 0.015	0.310 ± 0.004	0.249 ± 0.019	0.254 ± 0.018	0.227 ± 0.016	0.264 ± 0.016	0.216 ± 0.023	0.231 ± 0.005
L-Leucine	0.578 ± 0.012	0.579 ± 0.016	0.578 ± 0.010	0.457 ± 0.038	0.451 ± 0.037	0.417 ± 0.030	0.471 ± 0.030	0.377 ± 0.030	0.412 ± 0.010
L-Isoleucine	0.366 ± 0.008	0.363 ± 0.011	0.360 ± 0.006	0.248 ± 0.024	0.284 ± 0.020	0.259 ± 0.019	0.295 ± 0.019	0.238 ± 0.019	0.260 ± 0.008
L-Methionine	0.336 ± 0.008	0.345 ± 0.008	0.336 ± 0.007	0.274 ± 0.017	0.282 ± 0.023	0.245 ± 0.016	0.275 ± 0.018	0.222 ± 0.015	0.243 ± 0.007
L-Valine	0.874 ± 0.022	0.878 ± 0.024	0.891 ± 0.018	0.690 ± 0.018	0.691 ± 0.058	0.641 ± 0.051	0.733 ± 0.034	0.650 ± 0.051	0.703 ± 0.019
DL-Alanine	2.544 ± 0.054	2.464 ± 0.060	2.213 ± 0.155	2.054 ± 0.155	2.012 ± 0.238	2.004 ± 0.222	2.538 ± 0.171	2.308 ± 0.113	2.462 ± 0.017
L-Proline	2.885 ± 0.115	2.706 ± 0.130	2.625 ± 0.127	2.105 ± 0.111	2.165 ± 0.111	2.031 ± 0.244	2.127 ± 0.163	1.822 ± 0.212	1.786 ± 0.034
<u>Polar R groups</u>									
L-Threonine	1.344 ± 0.049	1.334 ± 0.127	1.259 ± 0.127	1.067 ± 0.276	1.063 ± 0.091	1.027 ± 0.083	1.187 ± 0.087	1.006 ± 0.104	1.124 ± 0.055
L-Serine	10.089 ± 0.442	9.450 ± 0.171	9.145 ± 0.559	7.936 ± 0.548	7.979 ± 0.418	7.537 ± 0.152	7.290 ± 1.121	7.152 ± 0.544	7.506 ± 0.211
L-Glutamine	3.653 ± 0.207	3.383 ± 0.244	3.364 ± 0.258	2.687 ± 0.281	2.702 ± 0.164	2.600 ± 0.178	2.904 ± 0.407	2.201 ± 0.185	2.191 ± 0.162
L-Asparagine	2.416 ± 0.173	2.562 ± 0.024	2.343 ± 0.206	1.994 ± 0.155	1.998 ± 0.181	1.929 ± 0.133	2.144 ± 0.166	1.567 ± 0.116	1.668 ± 0.038
Glycine	8.521 ± 0.206	8.335 ± 0.129	8.437 ± 0.105	5.626 ± 1.008	6.552 ± 0.605	6.514 ± 0.447	7.616 ± 0.497	6.692 ± 0.723	6.941 ± 0.174
Halfcystine	0.128 ± 0.005	0.128 ± 0.005	0.126 ± 0.002	0.114 ± 0.012	0.110 ± 0.010	0.110 ± 0.018	0.124 ± 0.012	0.082 ± 0.004	0.089 ± 0.004
Ornithine	0.012 ± 0.001	0.012 ± 0.001	0.015 ± 0.003	0.022 ± 0.006	0.017 ± 0.003	0.019 ± 0.008	0.022 ± 0.003	0.012 ± 0.002	0.014 ± 0.002
B-Alanine	2.514 ± 0.274	2.514 ± 0.274	2.330 ± 0.406	1.836 ± 0.186	1.872 ± 0.094	1.583 ± 0.183	2.039 ± 0.164	2.103 ± 0.153	2.252 ± 0.053

<sup>a</sup>Ninhydrin-positive compound



Table XIV  
Changes in the levels of ninhydrin-positive compounds (mM) in modified Grace's insect tissue culture medium by the growth and development of several stages of *Entomophthora egressa* isolate 521

NPC <sup>a</sup>	Time of incubation (h)								
	0	24	48	72	96	120	144	169	193
<u>Acidic</u>									
L-Aspartic acid	2.329 ± 0.122	2.546 ± 0.149	2.246 ± 0.531	2.401 ± 0.172	2.486 ± 0.140	2.372 ± 0.090	2.120 ± 0.199	1.824 ± 0.199	1.733 ± 0.080
L-Glutamic acid	3.411 ± 0.185	3.778 ± 0.184	3.552 ± 0.584	3.637 ± 0.290	3.838 ± 0.127	3.826 ± 0.168	3.254 ± 0.120	3.142 ± 0.331	3.351 ± 0.479
<u>Basic</u>									
L-Lysine	3.007 ± 0.163	3.273 ± 0.113	3.178 ± 0.689	3.131 ± 0.267	3.248 ± 0.116	3.252 ± 0.106	2.777 ± 0.230	2.783 ± 0.293	2.805 ± 0.064
L-Histidine	11.366 ± 0.178	12.664 ± 0.548	12.594 ± 0.862	11.582 ± 0.586	11.529 ± 0.443	12.146 ± 0.573	11.122 ± 0.535	11.516 ± 0.864	11.660 ± 0.233
L-Arginine	2.368 ± 0.124	2.601 ± 0.089	2.421 ± 0.586	2.471 ± 0.207	2.587 ± 0.081	2.561 ± 0.085	2.197 ± 0.180	2.185 ± 0.232	2.187 ± 0.054
<u>Nonpolar R groups</u>									
L-Tryptophan	0.453 ± 0.026	0.528 ± 0.051	0.432 ± 0.124	0.470 ± 0.038	0.477 ± 0.016	0.463 ± 0.014	0.416 ± 0.039	0.384 ± 0.040	0.404 ± 0.019
L-Phenylalanine	0.794 ± 0.045	0.864 ± 0.036	0.815 ± 0.195	0.826 ± 0.066	0.868 ± 0.028	0.724 ± 0.060	0.724 ± 0.060	0.674 ± 0.070	0.676 ± 0.017
L-Tyrosine	0.274 ± 0.018	0.301 ± 0.015	0.277 ± 0.064	0.283 ± 0.023	0.293 ± 0.011	0.266 ± 0.016	0.245 ± 0.017	0.212 ± 0.026	0.208 ± 0.007
L-Leucine	0.508 ± 0.030	0.555 ± 0.026	0.511 ± 0.121	0.524 ± 0.042	0.534 ± 0.020	0.471 ± 0.023	0.380 ± 0.021	0.276 ± 0.043	0.238 ± 0.011
L-Isoleucine	0.320 ± 0.022	0.351 ± 0.017	0.324 ± 0.077	0.331 ± 0.028	0.340 ± 0.013	0.310 ± 0.017	0.260 ± 0.014	0.212 ± 0.030	0.201 ± 0.007
L-Methionine	0.304 ± 0.022	0.330 ± 0.016	0.315 ± 0.026	0.311 ± 0.026	0.326 ± 0.011	0.280 ± 0.025	0.263 ± 0.011	0.234 ± 0.026	0.225 ± 0.003
L-Valine	0.760 ± 0.050	0.838 ± 0.044	0.790 ± 0.175	0.795 ± 0.068	0.836 ± 0.027	0.818 ± 0.030	0.698 ± 0.064	0.690 ± 0.074	0.676 ± 0.004
DL-Alanine	2.190 ± 0.129	2.414 ± 0.130	2.263 ± 0.501	2.287 ± 0.171	2.458 ± 0.088	2.402 ± 0.115	2.130 ± 0.282	2.145 ± 0.205	2.328 ± 0.085
L-Proline	2.532 ± 0.211	2.773 ± 0.136	2.736 ± 0.533	2.624 ± 0.208	2.724 ± 0.120	2.646 ± 0.125	2.208 ± 0.210	2.090 ± 0.214	2.208 ± 0.111
<u>Polar R groups</u>									
L-Threonine	1.192 ± 0.055	1.316 ± 0.074	1.201 ± 0.659	1.245 ± 0.066	1.284 ± 0.066	1.261 ± 0.052	1.142 ± 0.110	1.047 ± 0.100	1.077 ± 0.029
L-Serine	8.639 ± 0.304	9.448 ± 0.274	9.019 ± 0.626	8.780 ± 0.585	9.246 ± 0.207	9.443 ± 0.135	8.036 ± 0.560	7.588 ± 0.674	7.846 ± 0.288
L-Glutamine	3.180 ± 0.187	3.134 ± 0.090	2.942 ± 0.722	3.141 ± 0.150	3.322 ± 0.358	2.942 ± 0.084	2.751 ± 0.413	2.642 ± 0.280	2.571 ± 0.244
L-Asparagine	2.240 ± 0.122	2.468 ± 0.078	2.249 ± 0.585	2.373 ± 0.206	2.467 ± 0.094	2.351 ± 0.093	2.047 ± 0.161	1.791 ± 0.182	1.844 ± 0.091
Glycine	7.433 ± 0.320	8.111 ± 0.223	8.256 ± 0.172	7.677 ± 0.553	8.075 ± 0.211	8.146 ± 0.216	6.890 ± 0.628	6.686 ± 0.628	6.828 ± 0.160
Halfcystine	0.110 ± 0.010	0.122 ± 0.007	0.112 ± 0.010	0.131 ± 0.011	0.129 ± 0.004	0.100 ± 0.014	0.130 ± 0.008	0.092 ± 0.010	0.101 ± 0.006
Ornithine	0.018 ± 0.005	0.014 ± 0.001	0.018 ± 0.001	0.013 ± 0.002	0.015 ± 0.001	0.028 ± 0.004	0.015 ± 0.003	0.017 ± 0.003	0.016 ± 0.002
B-Alanine	2.095 ± 0.287	2.298 ± 0.310	2.334 ± 0.119	2.186 ± 0.204	2.431 ± 0.265	2.531 ± 0.228	1.535 ± 0.100	2.226 ± 0.198	2.091 ± 0.108

<sup>a</sup>Ninhydrin positive compound



during hyphal degeneration (169-193h).

Because of the different stages of development and the differing magnitudes of incidence of similar stages, a valid comparison between the isolates was to consider the level of change of NPC per 1000 protoplasts during the initial 48h of growth in MGM. Substantial differences were detected for all NPC except L-tryptophan, L-tyrosine, L-leucine, L-isoleucine, L-methionine, L-valine, L-glutamine, L-asparagine and D-alanine (Table XV). Isolate 458 appeared to require L-histidine, L-alanine, L-proline and DL-serine during this period; whereas, isolate 521 did not (Table XV). These NPC increased in concentration in the level of glycine.

(c) Total protein and glucose changes.

Depending on the stage of development both isolates either utilized proteins from or released proteins into the medium (Fig. 9). Protein release was detected for the early growth up to 24h for both protoplast isolates. Thereafter, only I521 continued to release protein during the protoplast phase (Fig. 9). Both isolates reduced the total protein level by a comparable 32% following the period of protein secretion. Isolate 458 and I521 achieved these reductions by 96h and 120h, respectively. For I521 the decline occurred during protoplast transformation; whereas, the decline for I458 occurred during protoplast growth and protoplast trans-



Table XV  
Changes in the levels of various NPCs<sup>a</sup>  
in MGM by the protoplast stage of both  
isolates<sup>b</sup> of Entomophthora egressa

NPC	Concentration (p moles/ml/1000 protoplasts) <sup>c</sup>				
		I521	I458	t-value	P-value
L-Aspartic acid	-	3.0 ± 0.2	- 13.5 ± 1.1	3.834	<0.01
L-Glutamic acid	+	5.1 ± 0.3	- 14.7 ± 1.2	4.569	<0.01
L-Lysine	+	6.2 ± 0.7	- 0.6 ± 0.9	4.114	<0.01
L-histidine	+	43.8 ± 1.1	- 28.4 ± 1.2	17.369	<0.001
L-Arginine	+	1.8 ± 0.1	+ 4.1 ± 0.3	2.277	<0.05
L-Tryptophan	+	0.8 ± 0.1	- 1.6 ± 0.7	1.386	>0.2
L-Phenylalanine	+	0.8 ± 0.2	- 3.1 ± 0.2	5.629	<0.001
L-Tyrosine		0.0 ± 0.0	+ 0.6 ± 0.3	0.816	>0.4
L-Leucine	+	0.1 ± 0.1	0.0 ± 0.0	0.407	>0.6
L-Isoleucine	+	0.1 ± 0.1	- 0.3 ± 0.1	1.155	>0.3
L-Methionine	+	0.4 ± 0.1	0.0 ± 0.0	1.633	>0.1
L-Valine	+	1.1 ± 0.1	0.8 ± 0.2	0.548	>0.6
L-Alanine	+	2.6 ± 0.2	- 15.0 ± 1.0	7.045	<0.001
L-Proline	+	7.3 ± 0.2	- 11.8 ± 1.1	6.974	<0.001
L-Threonine	+	0.3 ± 0.1	- 3.9 ± 0.7	2.425	<0.05
DL-Serine	+	13.6 ± 0.7	- 42.9 ± 1.3	15.622	<0.001
L-Glutamine	-	8.5 ± 0.1	- 12.3 ± 0.9	1.713	>0.1
L-Asparagine	+	0.3 ± 0.1	- 3.4 ± 1.1	1.368	>0.1
Glycine	+	29.4 ± 0.2	- 3.8 ± 0.3	38.337	<0.001
Halfcystine		0.0 ± 0.0	0.0 ± 0.0	—	—
Ornithine		0.0 ± 0.0	0.0 ± 0.0	—	—
B-Alanine	+	8.5 ± 0.1	8.5 ± 0.2	0	≈100

<sup>a</sup>Ninhydrin-positive compounds

cont.



## Table XV cont.

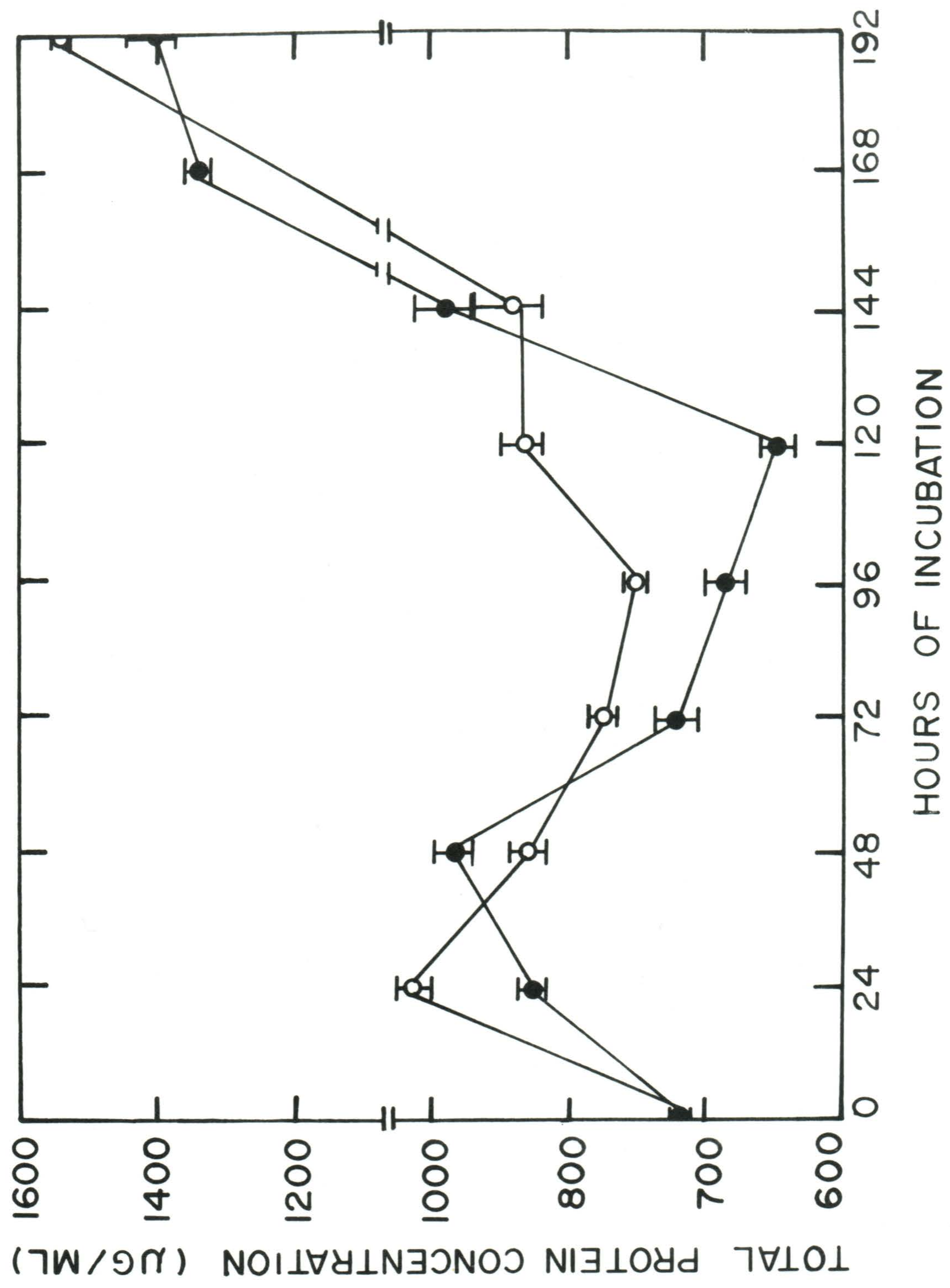
<sup>b</sup>Isolate 458 from L. fiscellaria fiscellaria

Isolate 521 from C. fumiferana

<sup>c</sup>Change in NPC in MGM after 48h incubation of protoplasts,  
n=6

Fig. 9. Change in the total protein levels of MGM during the development of Entomophthora egressa isolate 458 (○) and isolate 521 (●).





formation. During shb germination the protein levels rose in the medium by over 110%. Older fusion spheres of I458 also released protein into the medium.

Glucose was readily utilized by both isolates in most stages of development (Fig. 10). With the exception of samples at 96h the glucose levels declined continuously until 168h for both isolates. Isolate 458 increased in glucose consumption after 168h and I521 did not appear to use glucose after 168h. At 96h the level of glucose in MGM increased during cell wall regeneration of both isolates.

Because of heterogeneity in development and differences in magnitude of similar stages only the rate of glucose uptake per protoplast could be validly compared between isolates. Isolate 458 utilized glucose at a significantly lower rate than did I521 i.e.  $66 \pm 2 \text{ mg\%/h/1000 protoplasts}$  and  $78 \pm 3 \text{ mg\%/h/1000 protoplasts}$ , respectively ( $t=4.219$ ,  $P<0.01$ ).

(iii) Endogenous soluble NPC levels in 48h old protoplasts of both isolates. The levels of endogenous free soluble NPC out of 26 parameters showed conformity in concentrations for both isolates in that only L-proline, cystathionine and the identified total NPC differed between isolates (Table XVI). The major NPC (25 pico moles/1000 cells) included L-glutamic acid, L-histidine, L-alanine,



Fig. 10.      Glucose utilization during the growth of  
isolate 458 (○) and 521 (●) in MGM.

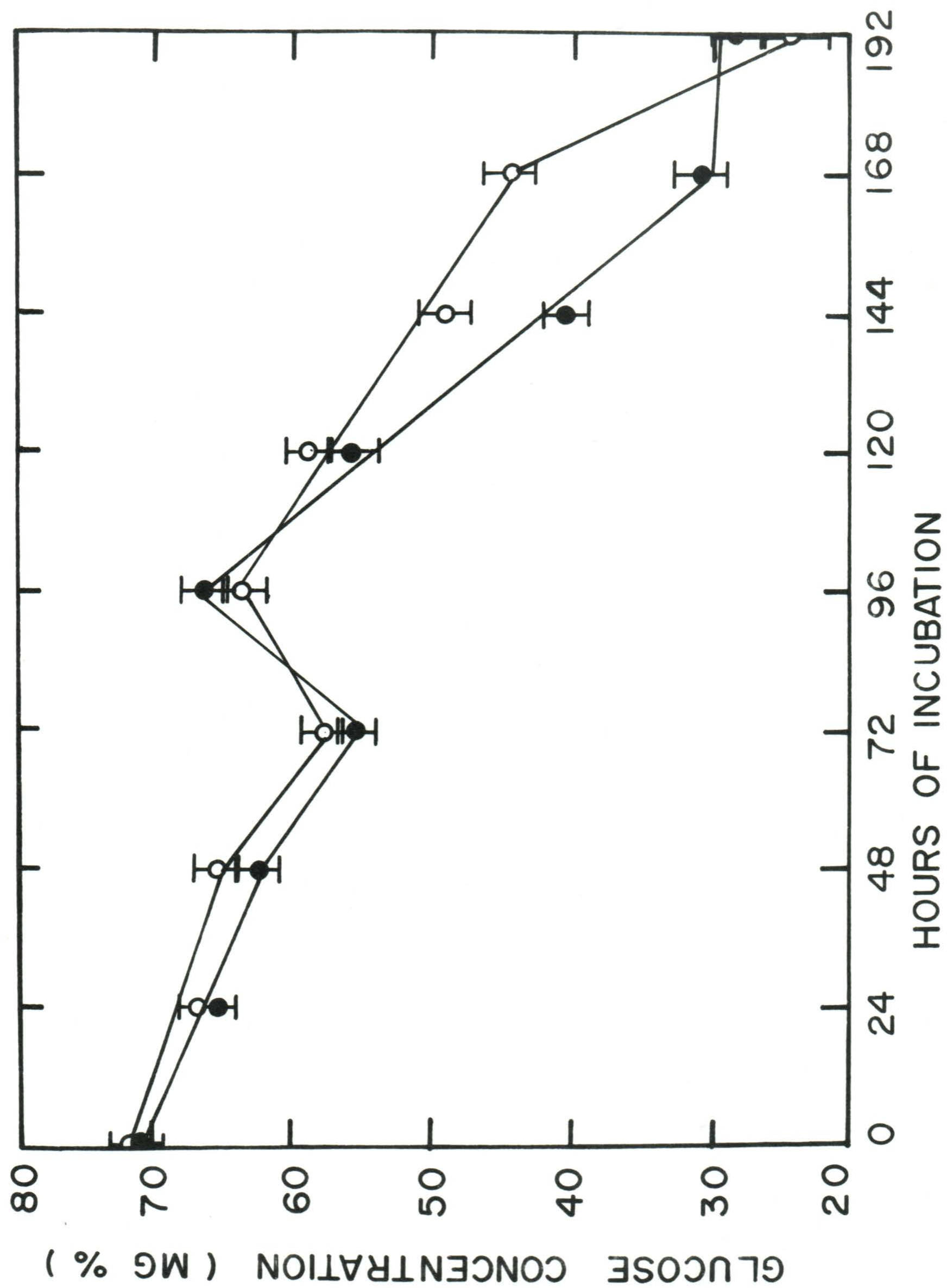




Table XVI  
Endogenous soluble ninhydrin-positive  
compounds in two isolates of the protoplast  
stage of Entomophthora egressa<sup>a</sup>

NPC	Concentration (p moles/1000 cells)			
	I521	I458	t-value	P-value
L-Aspartic acid	16.5 $\pm$ 1.3	13.1 $\pm$ 1.0	7.121	<0.001
L-Glutamic acid	37.4 $\pm$ 3.9	32.3 $\pm$ 2.9	0.555	>0.6
L-Lysine	35.4 $\pm$ 3.9	38.1 $\pm$ 2.9	0.229	>0.7
L-Histidine	54.7 $\pm$ 2.7	48.4 $\pm$ 0.8	0.913	>0.2
L-Arginine	11.3 $\pm$ 0.6	6.8 $\pm$ 3.6	0.503	>0.6
L-Tryptophan	1.6 $\pm$ 0.4	5.6 $\pm$ 4.0	0.406	>0.6
L-Phenylalanine	3.5 $\pm$ 0.4	2.5 $\pm$ 1.1	0.349	>0.6
L-Tyrosine	1.6 $\pm$ 0.2	1.0 $\pm$ 0.2	0.866	>0.4
L-Leucine	3.4 $\pm$ 0.3	2.4 $\pm$ 0.4	0.816	>0.4
L-Isoleucine	2.1 $\pm$ 0.4	1.4 $\pm$ 0.2	0.639	>0.5
L-Methionine	0.8 $\pm$ 0.2	1.2 $\pm$ 0.2	0.577	>0.6
L-Valine	3.4 $\pm$ 0.3	2.4 $\pm$ 0.2	1.155	>0.2
L-Alanine	27.3 $\pm$ 2.1	22.9 $\pm$ 1.9	0.898	>0.3
L-Proline	12.0 $\pm$ 2.0	7.0 $\pm$ 0.4	1.001	>0.2
L-Threonine	6.2 $\pm$ 1.0	6.6 $\pm$ 0.4	0.165	>0.8
L-Serine	50.8 $\pm$ 6.4	47.2 $\pm$ 1.2	0.226	>0.7
L-Glutamine	14.3 $\pm$ 2.3	11.5 $\pm$ 0.7	0.762	>0.4
L-Asparagine	7.5 $\pm$ 1.1	6.0 $\pm$ 0.2	0.548	>0.6
Glycine	43.8 $\pm$ 6.4	37.1 $\pm$ 0.6	0.426	>0.6
Halfcystine	0.6 $\pm$ 0.1	0.5 $\pm$ 0.5	0.134	>0.8
Ornithine	1.4 $\pm$ 0.2	0.4 $\pm$ 0.2	1.443	>0.1
B-Alanine	7.6 $\pm$ 0.7	6.9 $\pm$ 0.1	0.404	>0.1
Ethanolamine	1.0 $\pm$ 0.1	1.1 $\pm$ 0.4	0.099	>0.9

cont.

Table XVI cont.

NPC	Concentration (p moles/1000 cells)			
	I521	I458	t-value	P-value
Cystathionine	1.3 $\pm$ 0.1	0.2 $\pm$ 0.0	4.492	<0.01
$\gamma$ -Aminobutyric acid	1.0 $\pm$ 0.0	0.5 $\pm$ 0.3	0.680	>0.5
Total NPC	321.6 $\pm$ 2.9	281.2 $\pm$ 4.3	3.180	<0.01

<sup>a</sup>After 48h incubation in modified Grace's medium



DL-serine and glycine. Moderate levels (5-10 pico moles/1000 cells) were observed for L-aspartic acid, L-arginine, L-tryptophan, L-proline, L-threonine, L-glutamine, L-asparagine and B-alanine.

(iv) Endogenous soluble protein content in several fungal stages. The dry mass of the protoplast stage of I458 was greater than that of I521, whereas, the reverse situation occurred with the walled stages (Fig. 11). The dry mass of individual protoplast cells of I458 ( $17.3\mu\text{g}/\text{cell} \pm 0.2\mu\text{g}/\text{cell}$ ) was greater than the mass of the protoplasts of I521 ( $6.1\mu\text{g}/\text{cell} \pm 0.3\mu\text{g}/\text{cell}$ ;  $t=12.684$ ,  $P<0.001$ ).

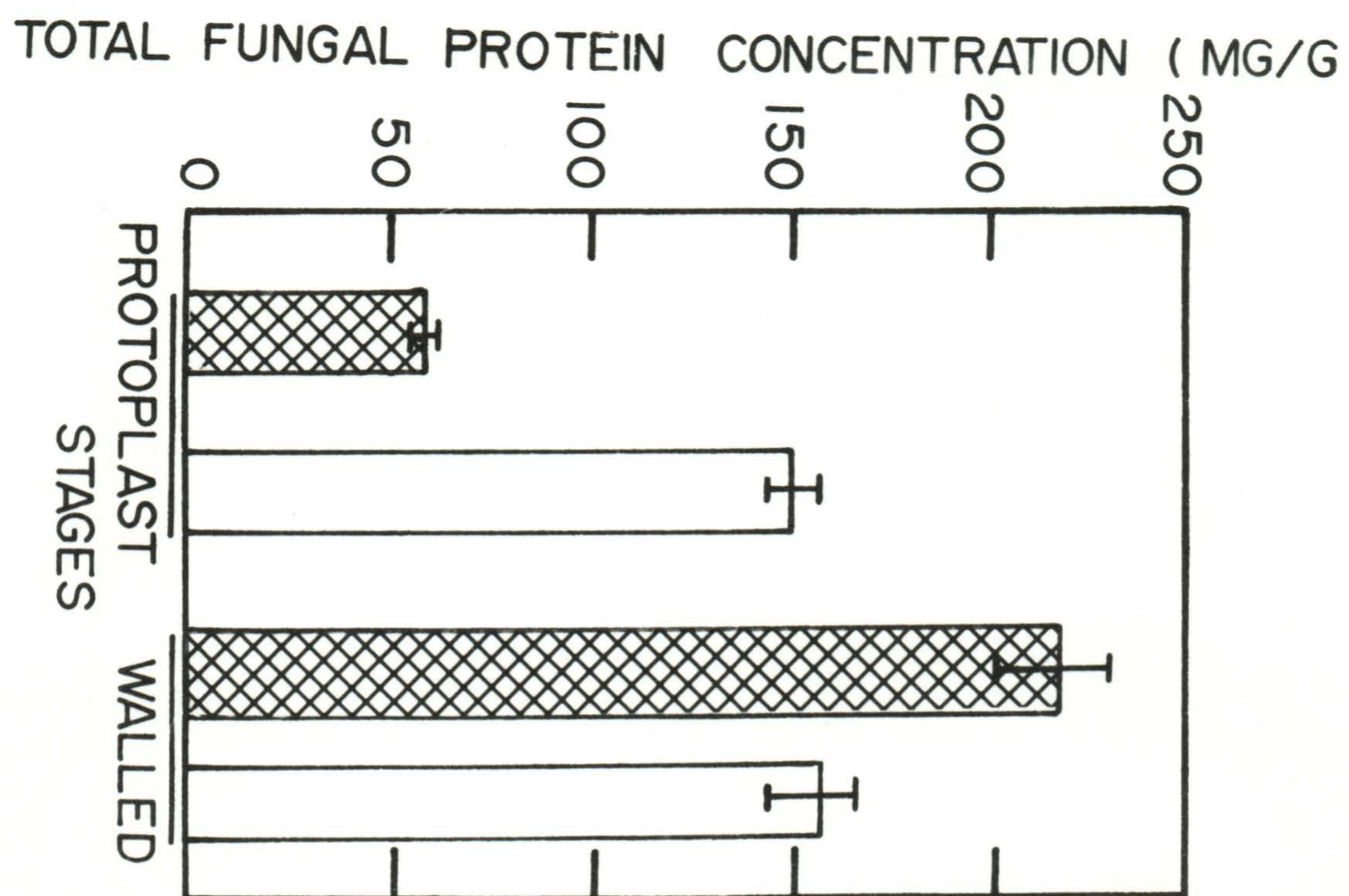
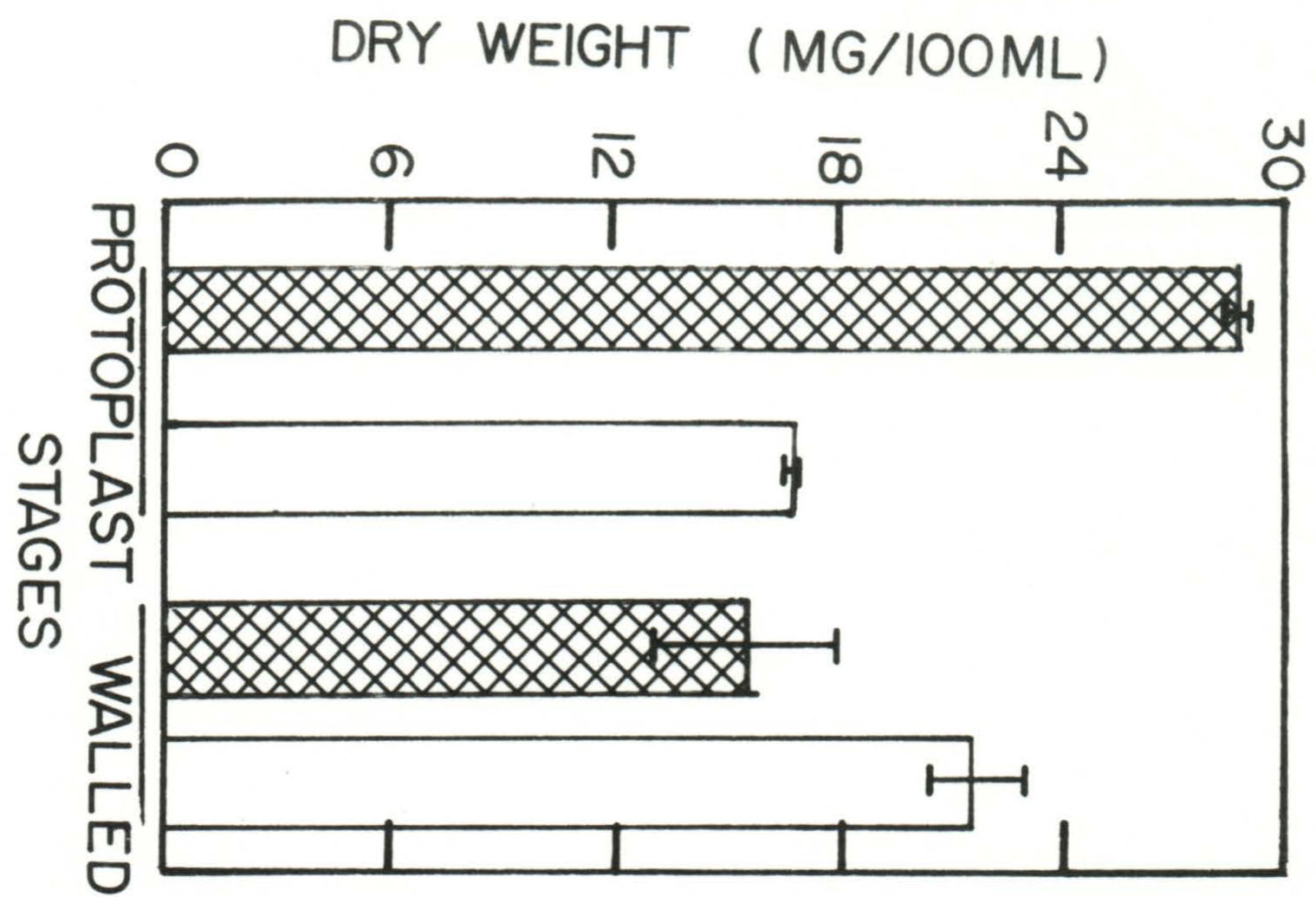
The endogenous soluble protein level of I458 protoplasts was less than that of the protoplasts of I521 (Fig. 12;  $t=13.98$ ,  $P<0.001$ ). The reverse pattern was observed with the walled stages (Fig. 12;  $t=6.121$ ,  $P<0.001$ ). The total protein content of I521 protoplast and walled stages were the same ( $t=0.626$ ,  $P>0.4$ ). The I458 protoplasts contained less protein than the fusion spheres (Fig. 12;  $t=15.206$ ,  $P<0.001$ ).

(v) Effects of carbon dioxide on fungal growth and development. Compared to the control samples, all levels of  $\text{CO}_2$  tested accelerated the growth of protoplasts of I458 during the initial 10h incubation period

Fig. 11. Dry weights of the protoplast and walled stages of isolate 458 (▣) and isolate 521 (||) of Entomophthora egressa in MGM.

Fig. 12. Protein concentration in the protoplast and walled stages of Entomophthora egressa isolates 458 (▣) and 521 (||) in MGM.





(Fig. 13). The generation times (GT) of all CO<sub>2</sub> treated cultures increased after 10h; and, with the exception of the 6%CO<sub>2</sub> samples, the GT were still less than the control value (Fig. 13; GT control=9.2h/generation, GT3%CO<sub>2</sub>=3.6h/generation, GT6%CO<sub>2</sub>=11.3h/generation, GT10%CO<sub>2</sub>=4.3h/generation). The optimum CO<sub>2</sub> level for maximum protoplast yields was 3% with the yield declining as the level of CO<sub>2</sub> increased.

The growth kinetics of I521 and its growth pattern responses to CO<sub>2</sub> differed from those of I458. Initially only 3% and 6% CO<sub>2</sub> levels accelerated the growth of the protoplasts above the control rate (Fig. 14). The 10% CO<sub>2</sub> level induced a decline in protoplast level. There were no signs of cell lysis. The growth rates of protoplasts in 3%CO<sub>2</sub> declined after 22.5h growth, the cells in 6%CO<sub>2</sub> declined in growth rate after 10h and the rate of protoplasts in 10%CO<sub>2</sub> increased after 10h. The optimum CO<sub>2</sub> level was 3%.

The levels of CO<sub>2</sub> did not influence the morphogenetic sequence previously described.

(vi) Effects of salts and sugars on the growth and morphology of *Entomophthora egressa*.



Fig. 13. Effect of 3% (○), 6% (◣) and 10% (Δ) CO<sub>2</sub> on the growth of protoplasts of isolate 458 of Entomophthora egressa compared to atmospheric levels (0.03%) (●).

PROTOPLAST CONCENTRATION (CELLS/ML  $\times 10^4$ )

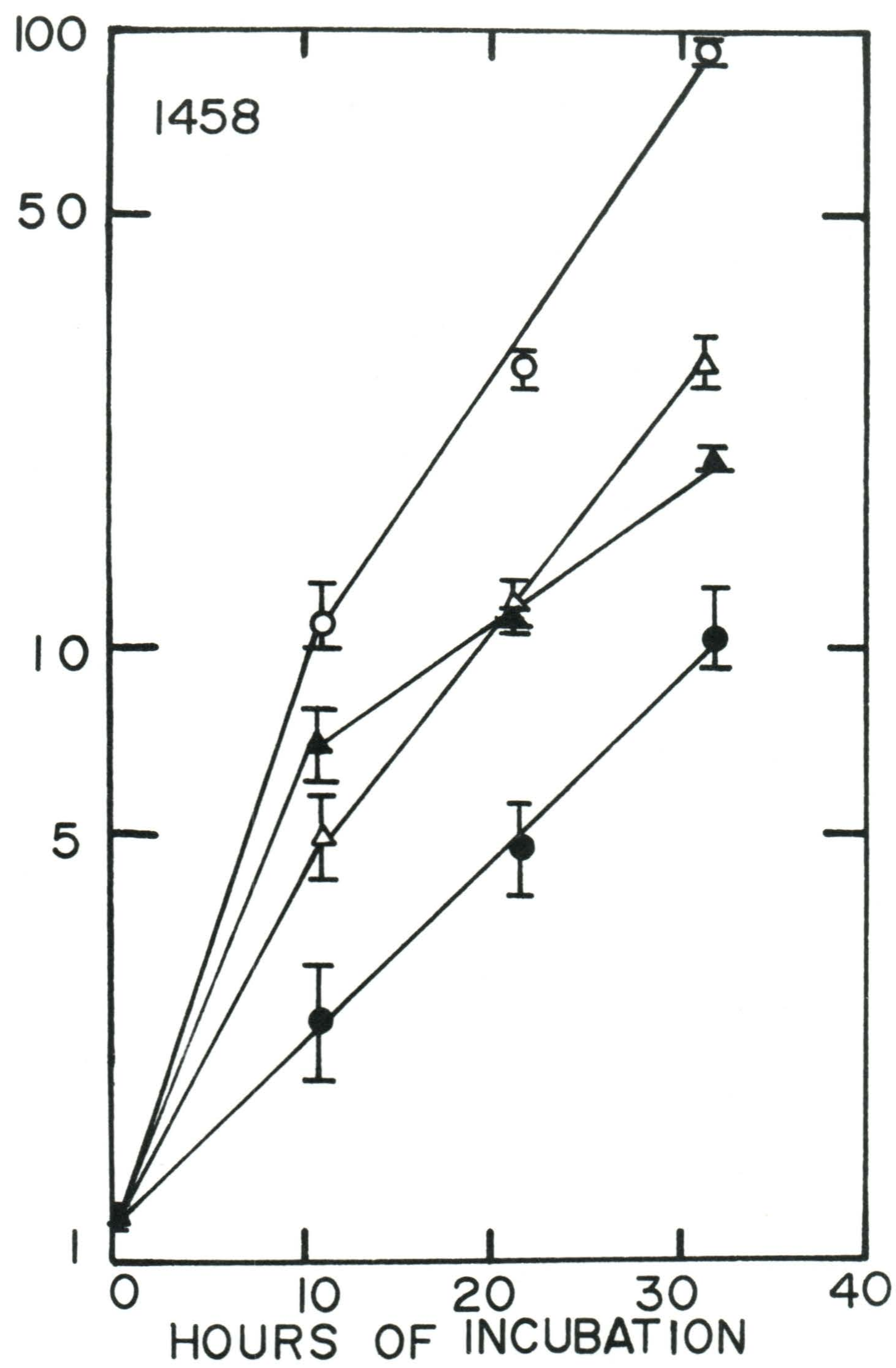
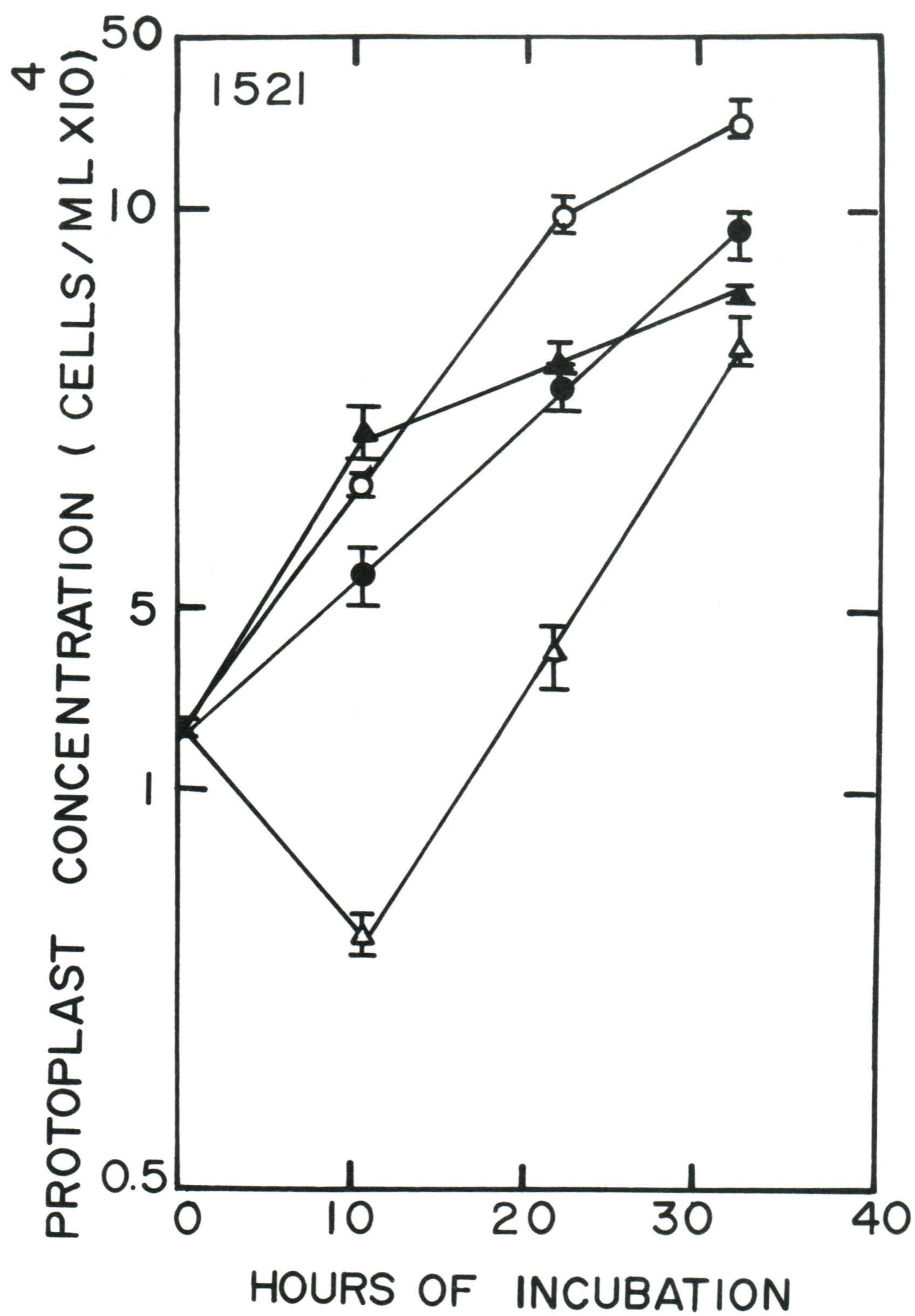




Fig. 14. Effects of 0.03% (●), 3% (○), 6% (▲) and 10% (△) CO<sub>2</sub> on the growth of Entomophthora egressa protoplasts of isolate 521.





(a) Growth kinetics. In medium A the protoplasts of I458 grew slowly up to 22.5h (GT=11.5h/generation) followed by a reduction in GT (GT=4h/generation) over the next 12h. Thereafter the GT increased to 17h/generation (Fig. 15). The cells in medium B had an 11h lag period followed by exponential growth (GT=3.5h/generation) until 22.5h and a decline in growth rate until 34.5h (GT=7.4h/generation). Thereafter, a stationary phase of 11h occurred (Fig. 15). Medium C favoured a long period of logarithmic growth (34.5h duration) with a GT of 7.5h/generation. After this period the GT of the protoplasts increased to 13.2h/generation followed by a plateau phase of 8.5h. During this plateau, the cells in medium A and B had entered the regeneration sequences by Dunphy and Nolan (1977b).

Isolate 521 generally exhibited longer generation times and different overall growth patterns (Fig. 16) than those of I458 (Fig. 15). The generation time (GT) of protoplasts in medium A, after an 11h lag period, was comparable to the GT during the early 22.5h growth period in medium B (GT in medium A = 6.5h/generation; GT in medium B = 6.0h/generation). In medium A the protoplast levels declined sharply after achieving a maximum concentration at 48h. The protoplasts in medium B grew more slowly after 22.5h (GT=20.0h/generation) until 48h and then declined at a rate

Fig. 15. Growth of protoplasts of isolate 458 of Entomophthora egressa in medium A (●), B (○) and C (▲).



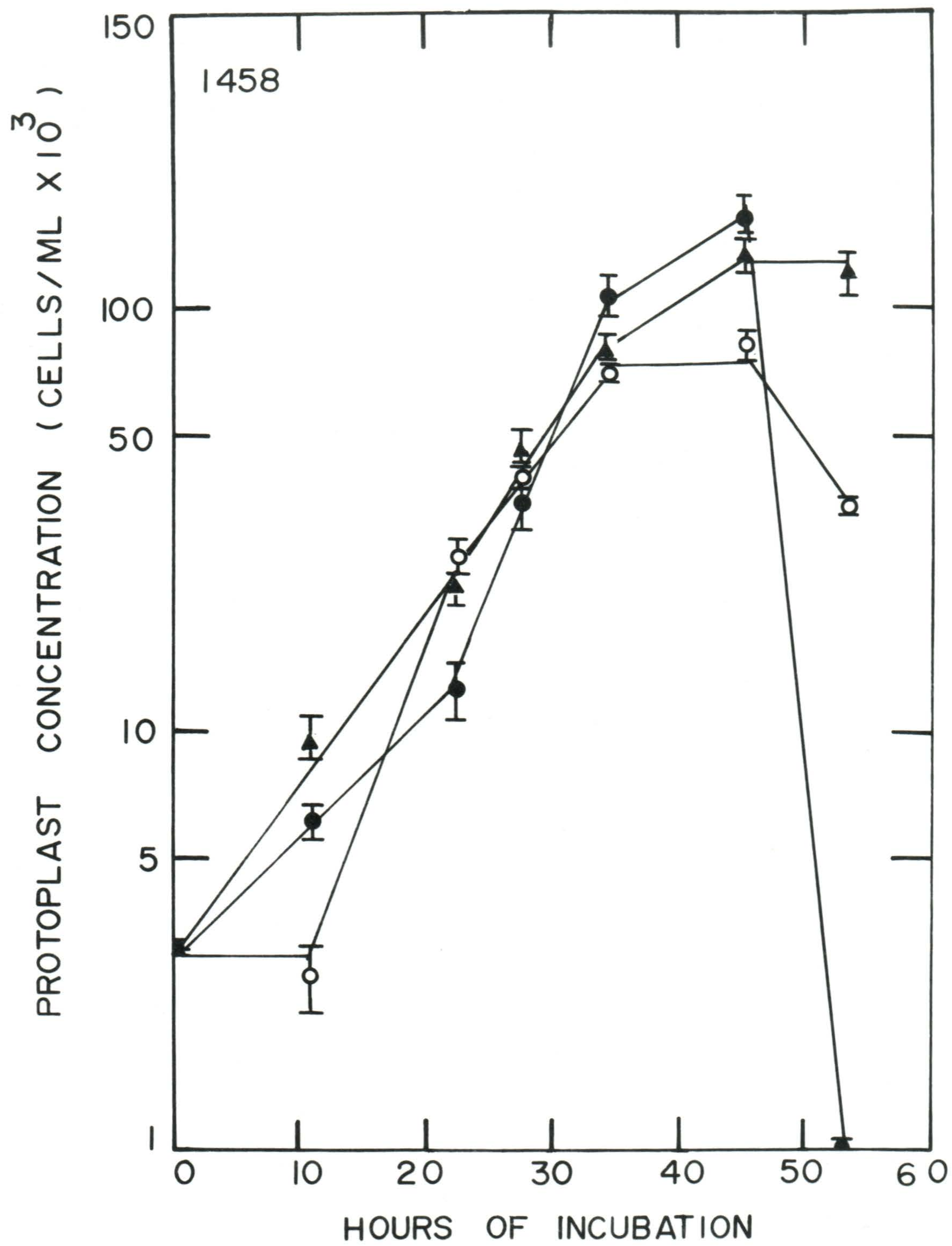
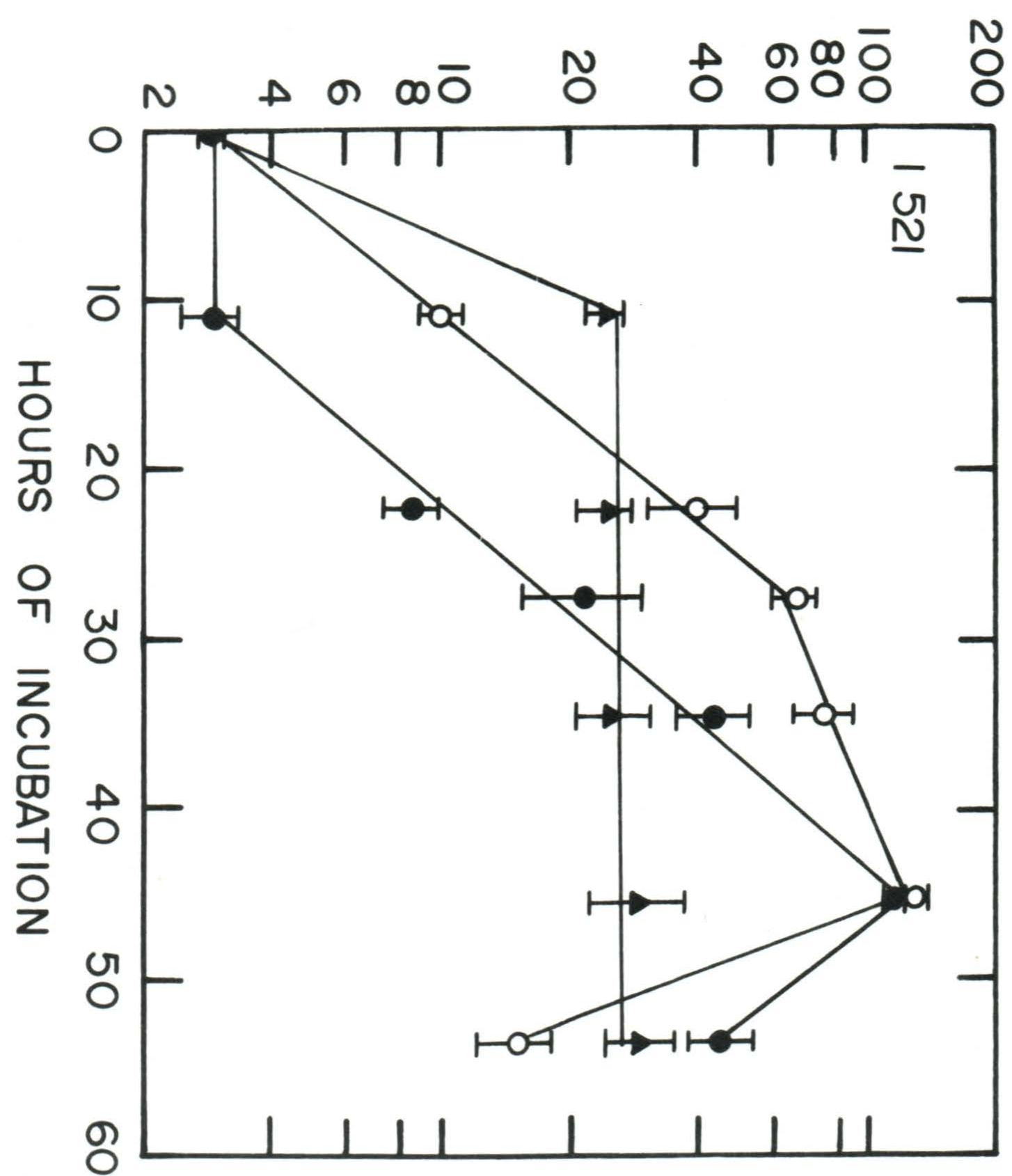


Fig. 16. Growth of Entomophthora egressa protoplasts of isolate 521 in medium A (●), B (○) and C (▲).



PROTOPLAST CONCENTRATION (CELLS/ML  $\times 10^3$ )



exceeding the decrease in medium A (Fig. 16). In medium C the protoplasts grew for 11h with the lowest GT (GT=3.8h/generation) followed by a long stationary phase (Fig. 16).

(b) Morphological kinetics. The various test media influenced the number of protoplasts comprising a chain (Figs. 17 and 18). There were no correlations between early growth rates and the number of protoplasts per chain for either isolate ( $r$  of I458=0.172,  $P$  0.8;  $r$  of I521=0.262,  $P$  0.7). This suggested that the effects of the media could be seen in the dissociation or inhibition of dissociation of the chains. Medium A, compared to all other test media, favoured the shortest chain length for both I458 and I521 (Figs. 17 and 18).

Medium A favoured a unimodal distribution for both isolates with greater protoplast numbers per chain occurring at 24.5h for I458 and at 34.5h for I521. Medium B favoured a bimodal profile for I458 and I521. Both initial maximum levels occurred at 10h with secondary peaks at 48.5h and 34.5h for I458 and I521, respectively. Medium C resulted in a unimodal distribution for I458 and a bimodal profile for I521 (Figs. 17 and 18).

The mesoproteoplasts existed during the growth phase at higher levels in medium A than did the mesoproteoplasts

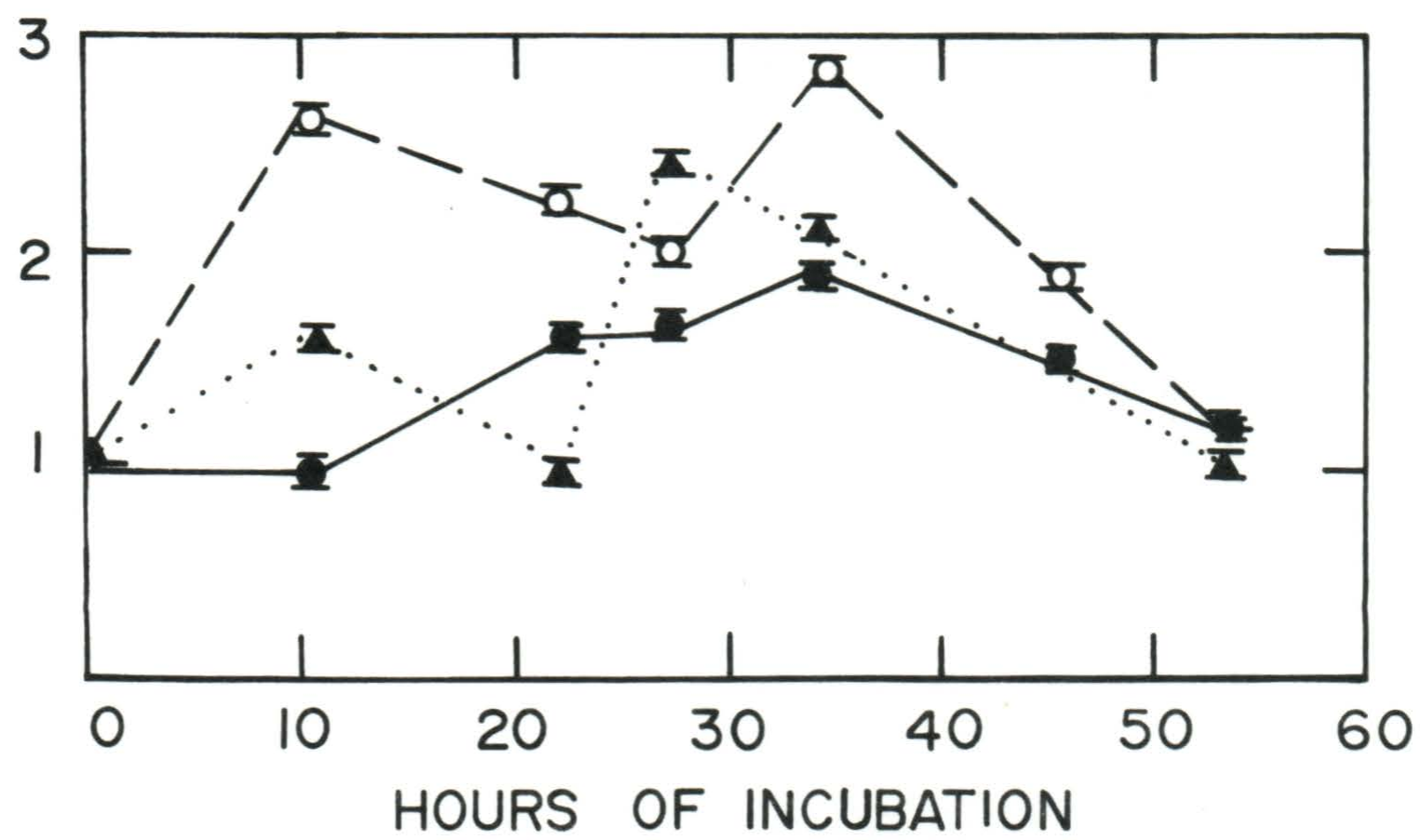


Fig. 17. Number of protoplast cells per chain for isolate 458 of Entomophthora egressa in medium A (●), B (○) and C (▲).

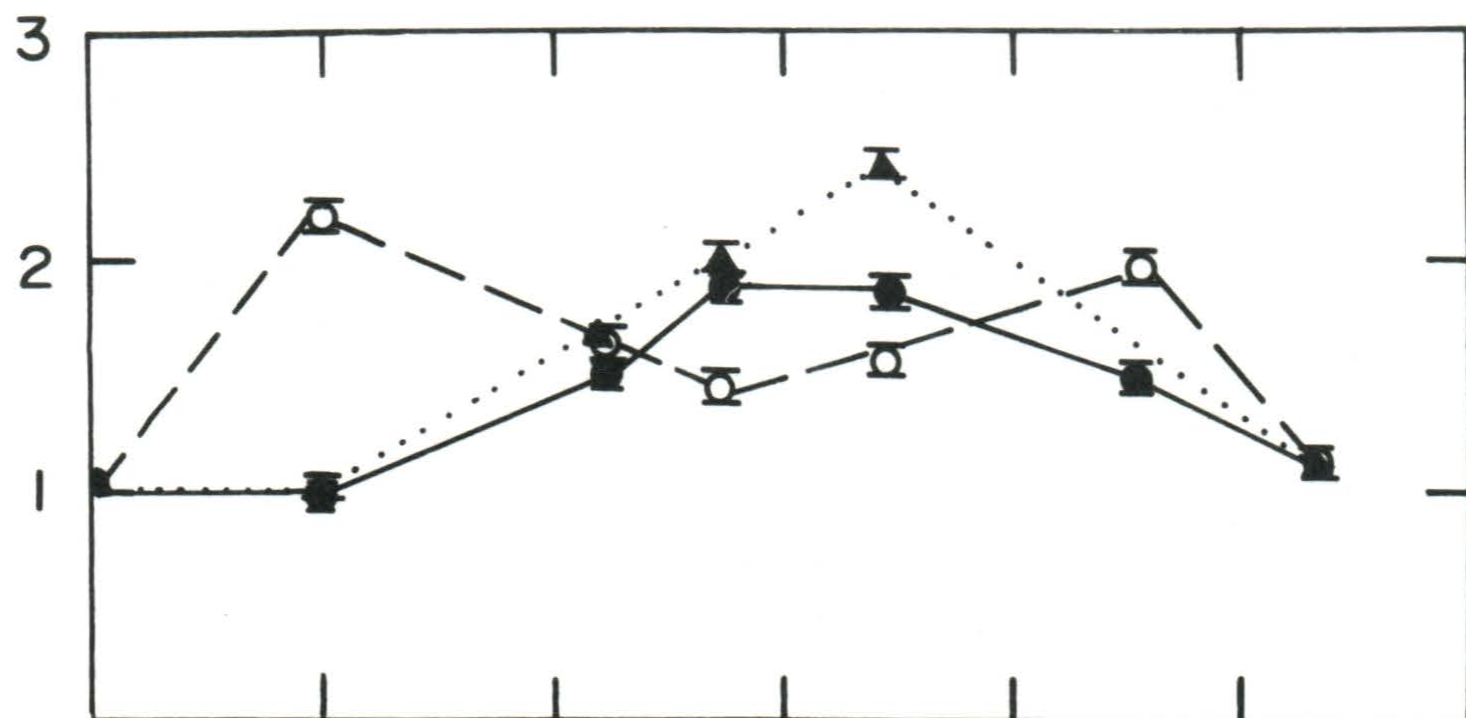
Fig. 18. Influence of medium A (●), B (○) and C (▲) on the number of protoplast cells per chain of isolate 521 of Entomophthora egressa.

NUMBER OF PROTOPLAST CELLS PER CHAIN

ISOLATE 521



ISOLATE 458





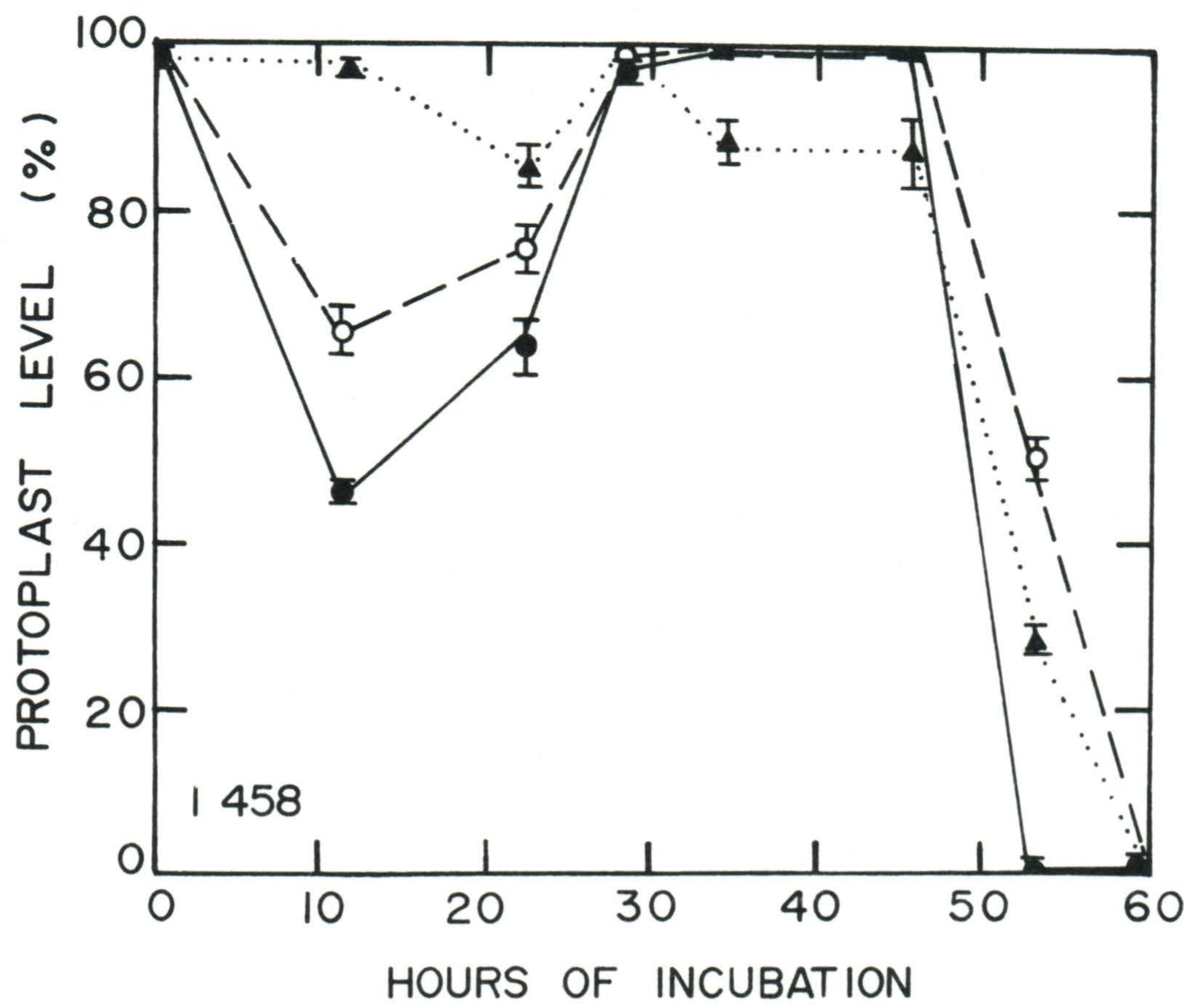
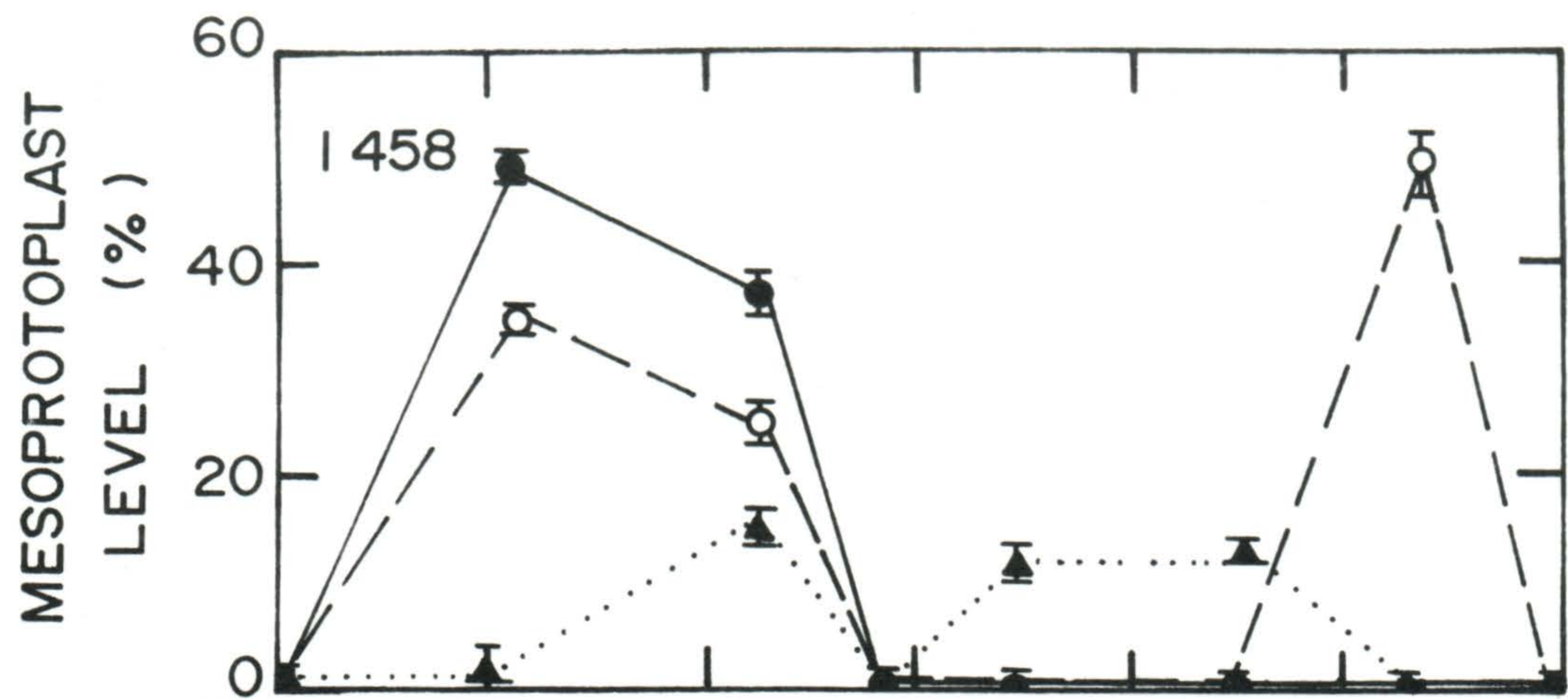
in the other media (Fig. 19). In medium B and C two phases of mesoprotoplasts were detected; one during active protoplast growth (10-30h) and the other during the plateau phases. In these media the plateau growth phase represented, in part, a balance between mesoprotoplast and protoplast reversion as evident from the reciprocal changes in these two stages (Fig. 20). The decline in protoplast levels at 50h (Fig. 20) also represented a reversion to the spherical hyphal body form. In medium A the plateau phase did not represent mesoprotoplast-protoplast transformations as evident by the absence of any reciprocity of these stages (Fig. 19). During the late plateau growth phase, a balance between protoplast division and spherical hyphal body formation occurred.

The distribution of mesoprotoplasts of I521 was unimodal for all test media (Fig. 21). In medium A and B the duration of the mesoprotoplast population of I521 was more contracted than that of I458. The mesoprotoplasts of I521 formed sooner in medium C than for I458 (Figs. 19 and 21). The three media induced higher levels of mesoprotoplasts of I521 than those of I458. Changes in the levels of mesoprotoplasts of I521, like those of I458 in the early growth period, reciprocated the levels of protoplasts (Figs. 20 and 22).

Fig. 19. Levels of the mesoprotoplast stage of isolate 458 of Entomophthora egressa in medium A (●), B (○) and C (▲).

Fig. 20. Levels of the protoplast stage of isolate 458 of Entomophthora egressa in medium A (●), B (○) and C (▲).





The absence of mesoprotoplasts during the duration of most stationary phases of growth of I521 protoplasts in medium C (Figs. 21 and 16) suggested that this medium stabilized the protoplasts which did not divide.

The incidence and rate of formation of spherical hyphal bodies were related to the type of medium and fungal isolate (Figs. 23 and 24). Isolate 521 exhibited a proclivity to form spherical hyphal bodies sooner but at a slower rate than I458 in a given medium.

In medium C I521 formed spherical hyphal bodies during the mid-point of the plateau in the growth curve (Figs. 23 and 16). The protoplasts began dividing during this period and maintained a constant absolute number as some cells reverted to the walled stages.



Fig. 21. Mesoprotoplast levels of isolate 521 of Entomophthora egressa in medium A (●), B (○) and C (▲).

Fig. 22. Protoplast levels of isolate 521 of Entomophthora egressa in medium A (●), B (○) and C (▲).

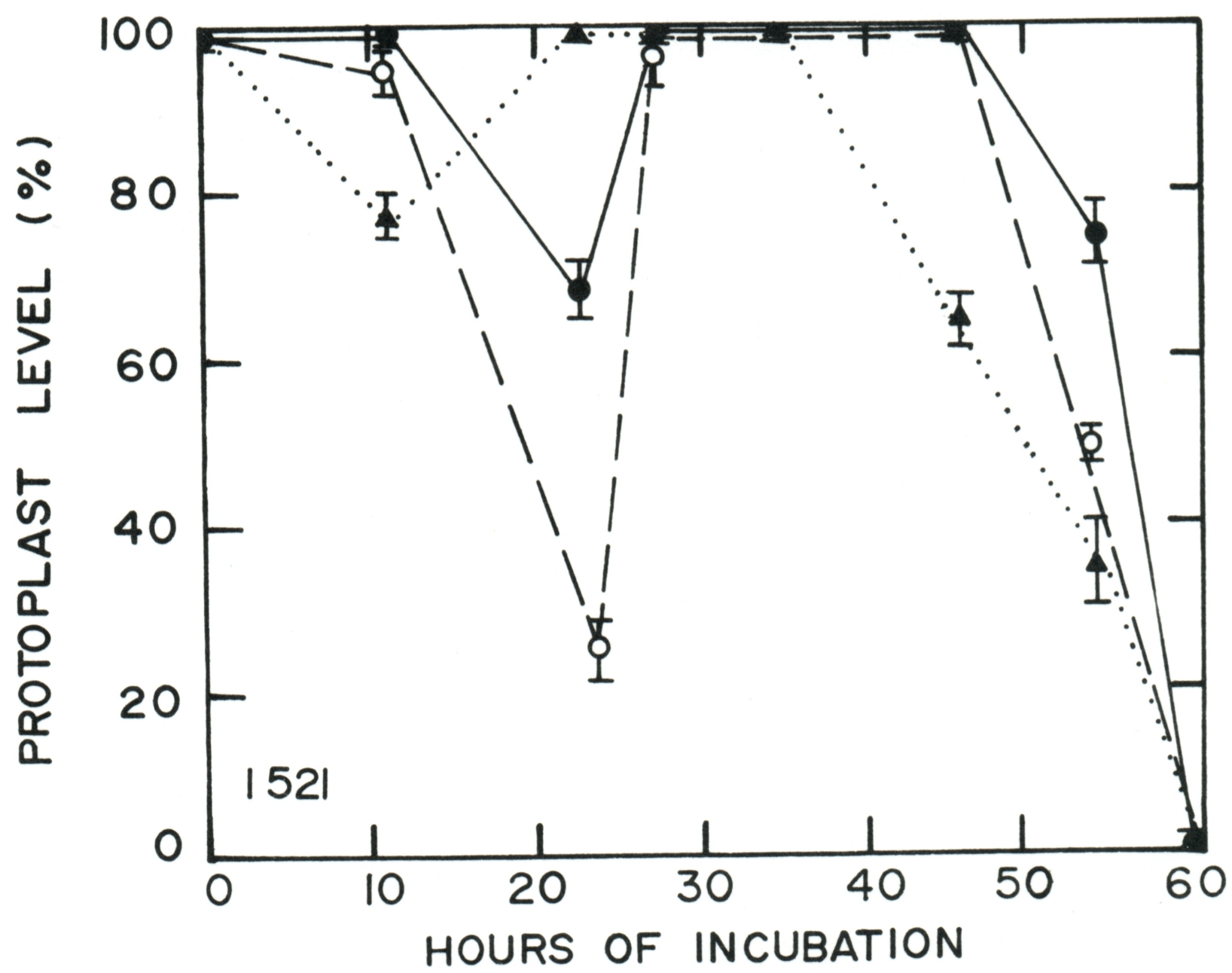
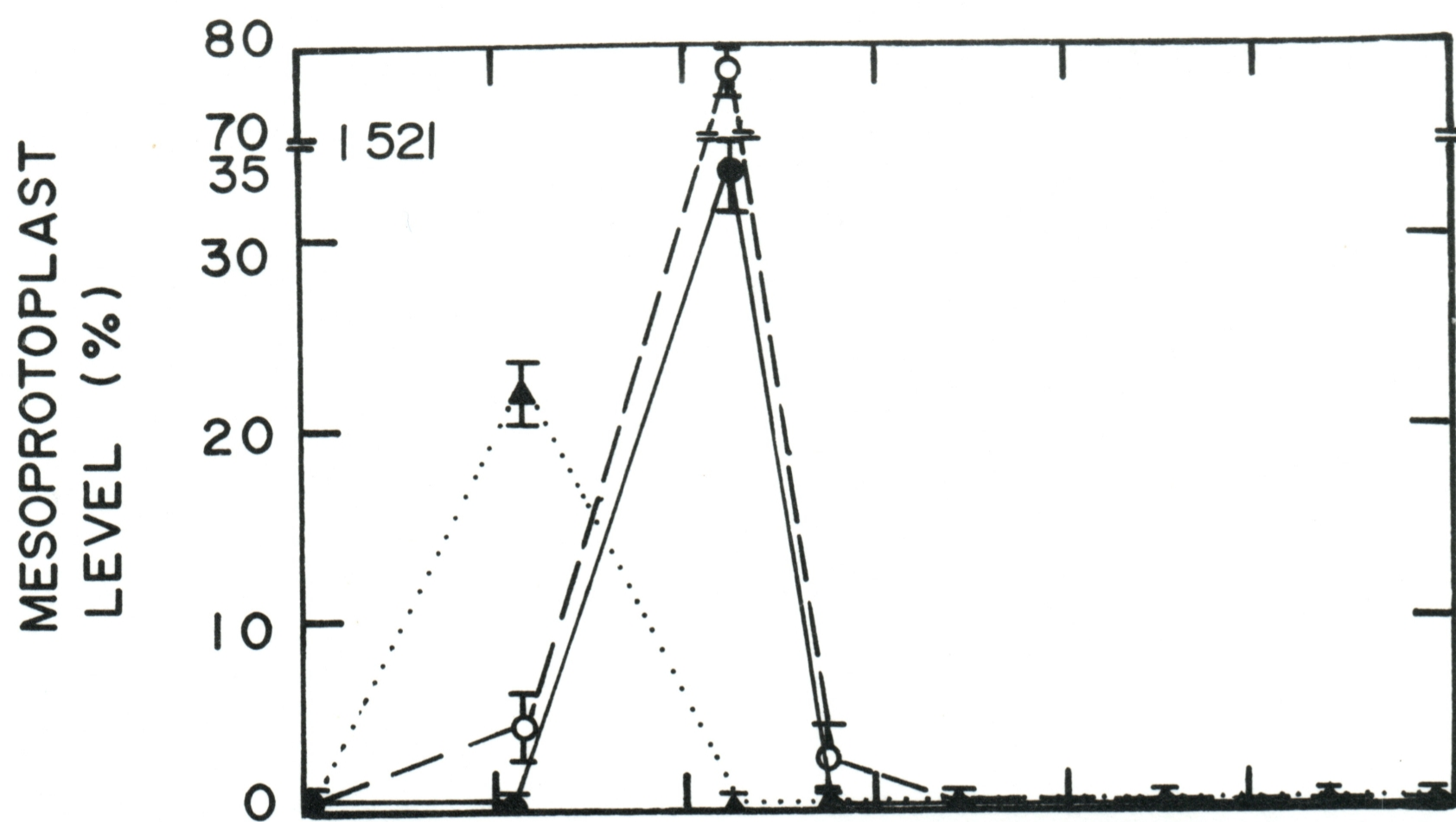




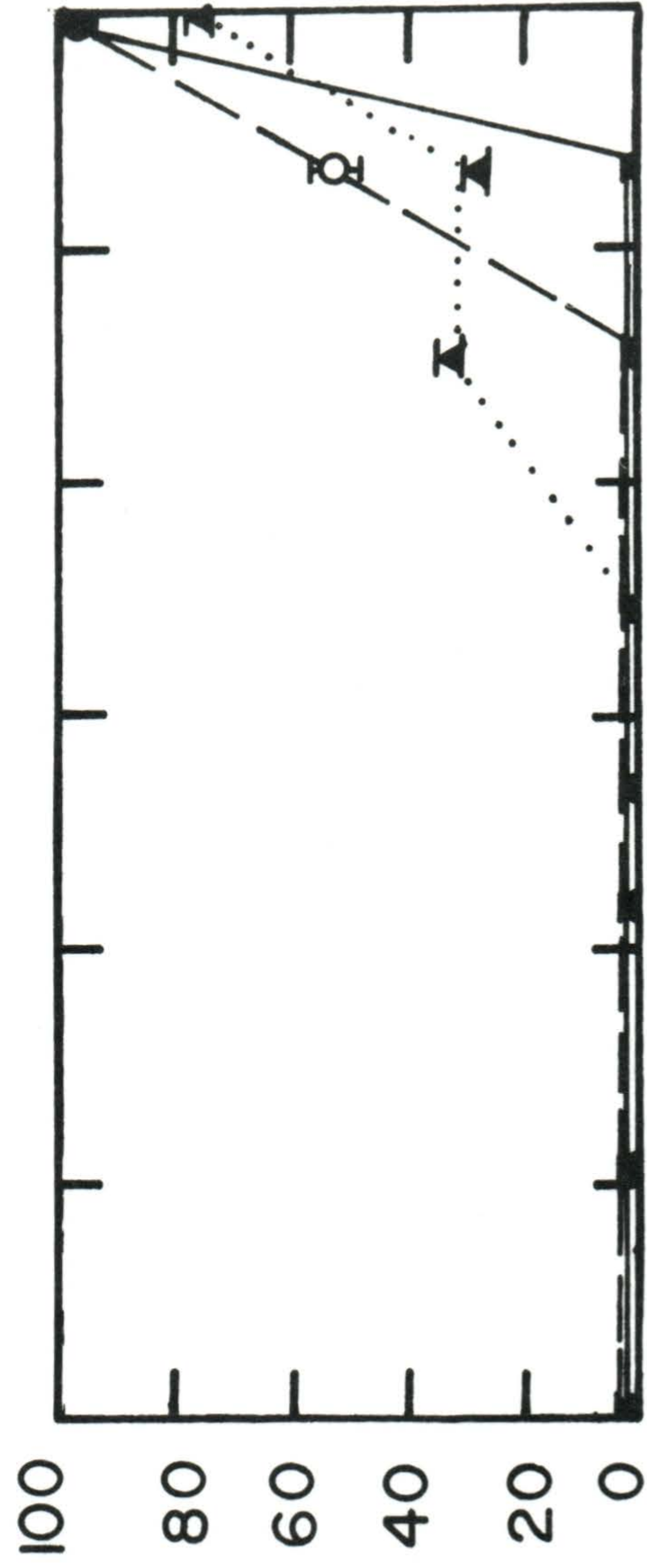
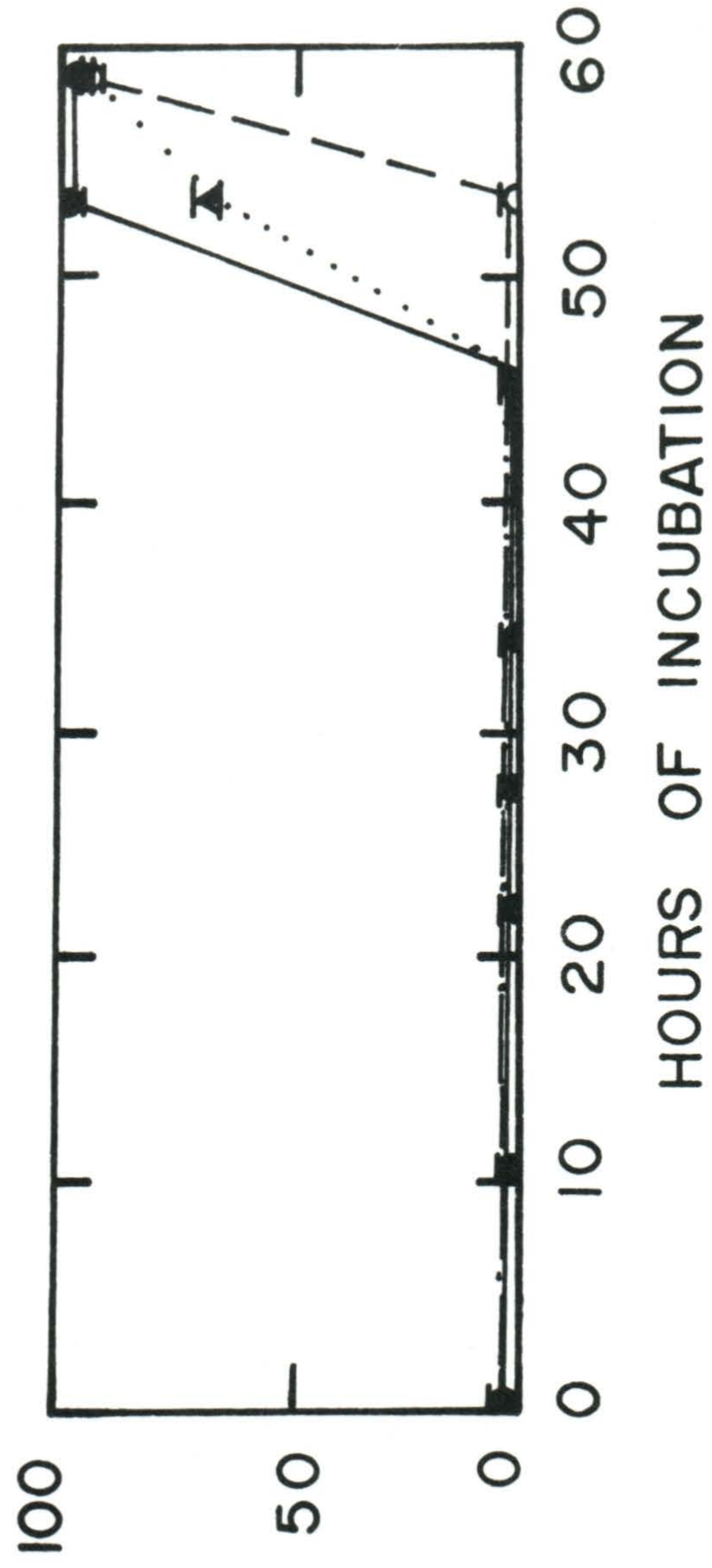
Fig. 23. Levels of the spherical hyphal bodies of Entomophthora egressa isolate 521 in medium A (●), B (○) and C (▲).

Fig. 24. Levels of the spherical hyphal bodies of Entomophthora egressa isolate 458 in medium A (●), B (○) and C (▲).

SPHERICAL HYPHAL BODY LEVEL (%)

ISOLATE 458

ISOLATE 521





B. Discussion. The comparison of mycelial development of E. egressa on coagulated egg yolk medium suggested that the two isolates were physiological races.

Studies of protoplast growth and regeneration in selected media enhanced the physiological race concept. The protoplasts of isolate 458 developed in a totally different fashion than those of I521 in MGM. In the simplified version of this medium (see Dunphy and Nolan 1977b), the rates of protoplast growth differed and while the regeneration sequences were similar, they differed in the levels of each stage and the rates of development. Dunphy et al. (1978) reported similar results for 2 isolates of E. egressa obtained from spruce budworm larvae at different geographical sites. Differences in colony morphology and growth rates of P. farinosa isolated from individual L. decemlineata have been reported by Bajan et al. (1971).

In medium A, B and C the levels of each stage of development and their formation time was isolate variable. Medium C, which contained more fructose and less glucose than medium A or B, suppressed the mesoprotoplast stage and did not prolong the duration of the protoplasts of I458 as opposed to I521, after maximum protoplast growth, to the same extent as did the other media. The low level of  $\text{Ca}^{+2}$  and the high level of  $\text{K}^{+}$  prolonged the protoplasts stage and influenced the



levels of mesoprotoplasts to a greater and lesser extent, respectively. The effects also varied with the fungal isolate. Sietsma and De Boer (1966, 1973) reported that inorganic salts completely inhibited the regeneration of Pythium sp.; whereas, sucrose and other organic compounds did not. The mechanism for the effects of the osmotic stabilizers on fungal protoplasts remains unknown (Villanueva 1966, Peberdy 1979). That a change in the level of  $\text{CaCl}_2$  and/or  $\text{KCl}$  produced the same results for a given isolate in a medium series suggested that these components were initially present at levels in excess of the needs of a given isolate. This argument may also apply to the experiments dealing with changes in the carbohydrate levels.

The response of both isolates to different levels of  $\text{CO}_2$  was interesting in that the  $\text{CO}_2$  levels in the hemolymph of several species of Lepidoptera have been reported to range from 4% to 6% (V/V, Roeder 1953). This suggests that the response by protoplasts of E. egressa to  $\text{CO}_2$  may represent an adaptation by the fungus to the hemolymph of the spruce budworm larvae. Although both isolates revealed an optimum  $\text{CO}_2$  level of 3%, the differences in growth kinetics in conjunction with the growth results in various types of media reveal that both isolates differ in metabolic aspects which may be translated into differences in pathogenicity. These aspects will be expanded on later in the text.



The levels of CO<sub>2</sub> did not influence the morphology of either protoplast isolate. Various levels of CO<sub>2</sub> have been found to stimulate the growth of Blastocladiella pringsheimii Reinsch (Cantino 1949), Blastocladiella emersonii Cantino and Hyatt (Cantino 1956), Macrochytrium botrydioides Minden (Crasemann 1954) and Sclerotium rolfsii Sacc. (Kritzman et al. 1977). CO<sub>2</sub> has been reported to influence the vegetative or reproductive phases of Mucor rouxii (Colmette) Wehmer (Bartnicki-Garcia and Nickerson 1962, Bartnicki-Garcia 1968) and Histoplasma capsulatum Darling (Salvin 1949) but did not influence Zygorhynchus viullemirii Namyel and Mucor ramannianus Moll. (Tabak and Cooke 1968). Tabak and Cooke (1968) reviewed the ability of fungi to fix CO<sub>2</sub> into tricarboxylic acid cycle intermediates and keto-acids for amino acid synthesis.

The growth rates and patterns suggested interisolate metabolic differences between the protoplast isolates; and the changes in medium pH, osmolality, total and individual NPC of the medium, protein concentration and glucose utilization add credence to this proposal. These differences argue for genetic differences in protoplast adaptability to the various media and enhance the initial proposal of II. B. 2. that although the protoplasts may grow and develop in the host hemocoel the isolates may not be equally adapted to the same host.



A comparison of the change in the concentration in the medium of a given amino acid per 1000 protoplasts revealed that I458 utilized more of the NPC than did I521. The data suggests, in view of the similarities in the endogenous free amino acid pools of both isolates, the possibility of different uptake mechanisms and/or different metabolic pathways.

Pelletier and Keitt (1954) and Campbell et al. (1978) have speculated that interisolate variability of entomopathogenic fungi in amino acid utilization may be related to pathogenicity and host specificity.

The relative levels of the major NPC in lysates of E. egressa were similar to those of Phycomyces nitens Kurze and Thaminidium elegans Link (Whitehead et al. 1960), Cunninghamella elegans Lender (Lin et al. 1975) and Phycomyces blakesleeanus Burget (Cohen and Farnham 1976). The presence of glutamine in E. egressa protoplasts may be indicative of nitrogen storage utilization and the synthesis of chitin. The high levels of L-glutamic acid, L-alanine, L-serine and glycine may be indicative of a transamination reactions. Those amino acids found in high concentration in the protoplasts are also the more concentrated NPC in the spruce budworm hemolymph (refer to section IX. A. 1.), which suggests the parasite has evolved with the host.



The differing protoplast yields and the different masses between protoplasts (section A. 2. iv.) accounted for the differences in fungal protoplast dry mass per 100ml. The increase in dry mass of the walled stage of I521 was a reflection of the germination of the spherical hyphal bodies. The decline in mass of I458 during fusion sphere formation may represent a period of excess energy expenditure during cell wall formation.

In view of the secretion of proteins by the spherical hyphal bodies of I521 and the fusion spheres of I458, the increase in fusion sphere endogenous soluble protein over the protoplasts of I458 and the constant soluble protein levels in the protoplasts and spherical hyphal bodies of I521 it would appear that I458 was more active in protein synthesis than I521.

The decline of glucose during most of the stages of fungal development confirms the use of glucose by E. egressa for carbon and energy as reported by Dunphy (1977). With the exception of species of Basidiobolus, the majority of the Entomophthorales grow and sporulate very well on media containing glucose (Latge 1975).

The increase in glucose level during the period of cell wall formation in MGM by both protoplast isolates may



reflect the release or leaching of glucose into the medium or the leaching away of glucose from the sites of cell wall synthesis. Necas (1971) proposed the latter mechanism to explain the inability to complete cell wall regeneration by protoplasts of Saccharomyces cerevisiae Hansen in liquid media.

The fusion sphere initials and spherical hyphal body initials of I458 and I521 in MGM and the spherical hyphal body initials of I458 in medium A, B and C show strong morphological similarities. In view of the similarities in protein secretion and glucose utilization, it would appear that the fusion spheres may be improperly developed spherical hyphal bodies inhibited in MGM. This would accentuate the divergence of genetic expression between the two isolates of E. egressa.

The nutritional variation present among isolates of E. egressa has counterparts among isolates of Basidiobolus meristosporus Eid. and Entomophthora coronata (Cost.) Kevorkian (Latge 1975a), Entomophthora obscura Hall and Dunn (Latge 1975b), Entomophthora phalloides Batko and Entomophthora aphidis Hoffman (Latge et al. 1978). Claydon (1978) reported that 2 strains of Entomophthora virulenta Hall and Dunn grew to different levels for a given level of glucose. Physiological diversity as expressed in terms of



speed of growth on solid media, ability to sporulate and the diversity in ability to infect spruce budworm larvae have been reported for Entomophthora sphaerosperma Fries (Kenneth 1978).

## V. Analysis of granular cell and protoplast cell membranes.

The granular hemocytes of either host insect failed to adhere to either protoplast isolate. To explore the possibility of the protein components of the surface of the protoplasts preventing hemocyte adhesion the protoplasts of either isolate were exposed to selected proteolytic enzymes. These enzymes were also used to study the nature of the hemocyte surfaces.

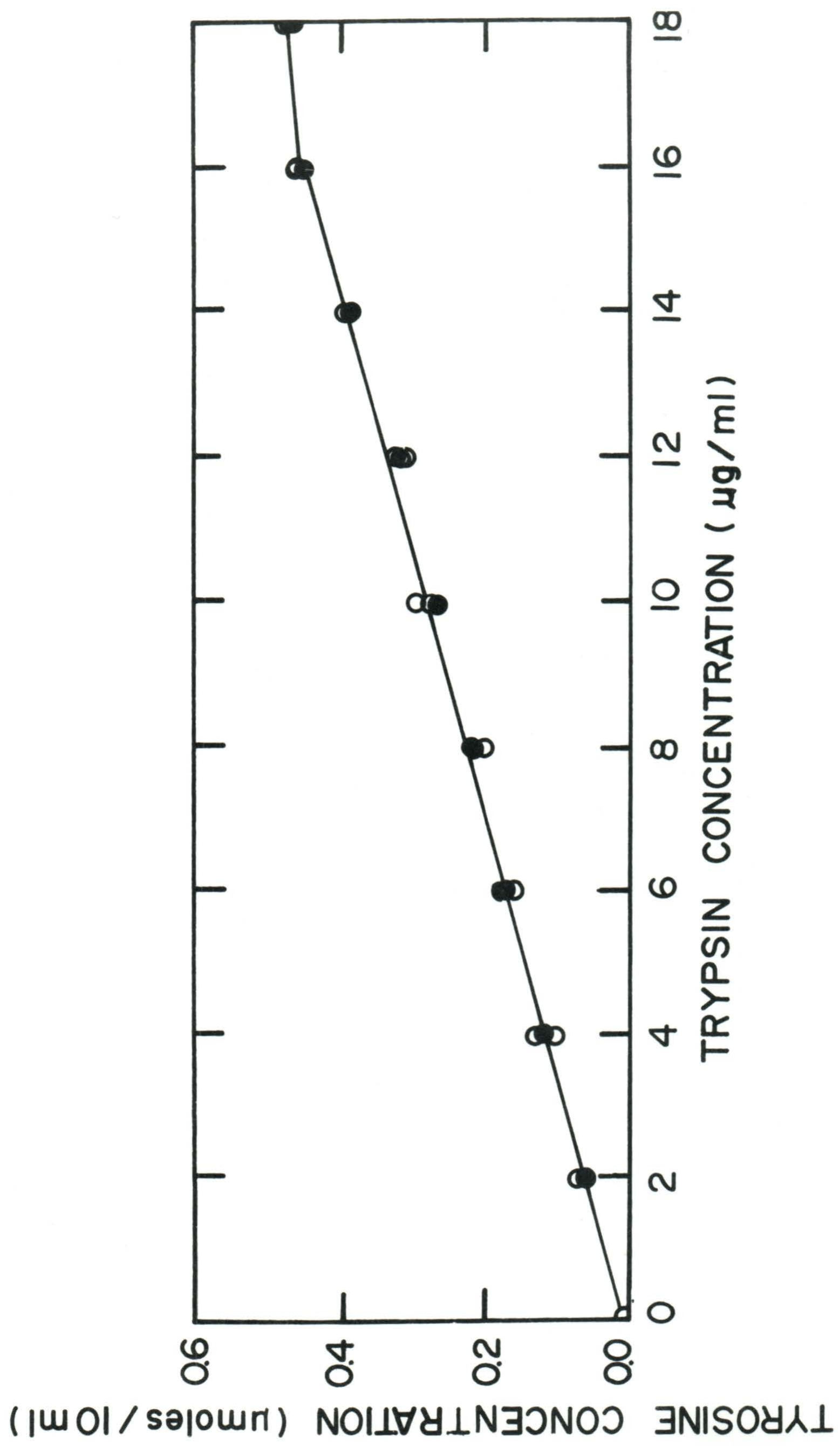
### A. Results.

#### 1. Trypsin study.

(a) Trypsin assays. The tyrosine release profiles (Fig. 25) were identical for trypsin in the assay mixture with and without added sucrose. The trypsin activity based on the trypsin unit (TU) was the same for both enzyme assays with  $48.76\mu\text{g}/\text{TU} \pm 0.62\mu\text{g}/\text{TU}$  and  $50.02\mu\text{g}/\text{TU} \pm 0.73\mu\text{g}/\text{TU}$  for the substrate solution and substrate solution with sucrose, respectively ( $t=1.316$ ,  $P>0.2$ ). Because sucrose did not inhibit trypsin activity, it was the osmostabilizer chosen to sustain the protoplasts. The activity of trypsin in a filter sterilized solution was the same as the activities indicated above ( $49.75\mu\text{g}/\text{TU} \pm 0.83\mu\text{g}/\text{TU}$ ,  $t=0.714$ ,  $P>0.4$ ). It would be possible, consequently, to conduct growth curve



Fig. 25. Release of tyrosine from hemoglobin using trypsin with (○) and without (●) sucrose. Each circle represents the mean of three replicates.





studies of trypsinized protoplasts using aseptic conditions.

(b) Protoplast viability. Exposing protoplasts of isolate 521 to 1TU influenced both the growth rate of the protoplasts and the final cell yields. Protoplasts exposed to trypsin for 0, 10, 20, 30 and 40min produced growth rates of 5.1, 4.2, 5.5, 3.8 and 4.0h/generation, respectively (Figs. 26 and 27). The 10 and 20min exposure times dramatically reduced the protoplast yields below that of the control, whereas, protoplasts exposed to trypsin for 30 and 40min yielded levels comparable to the control levels.

To assist in determining an optimum exposure time of protoplasts to trypsin the effects of introducing trypsin to protoplast suspensions were assessed in terms of final protoplast concentration, percentage of spindle shaped protoplasts and the release of tyrosine from the protoplasts. As the exposure time increased the amount of tyrosine increased up to 20min and leveled off thereafter (Fig. 28). The percentage of spindle shaped protoplasts declined throughout the study time. The level of protoplasts/ml remained constant until 30min after which a 20% reduction occurred (Fig. 28). The medium was very granular during this period indicating protoplast lysis.

Fig. 26. Growth of protoplasts of Entomophthora egressa isolate 521 after exposure to 1 trypsin unit for 0 (●) and 10 (○) minutes.

Fig. 27. Growth of protoplasts of Entomophthora egressa isolate 521 after exposure to 1 trypsin unit for 20 (●), 30 (▲) and 40 (○) minutes.



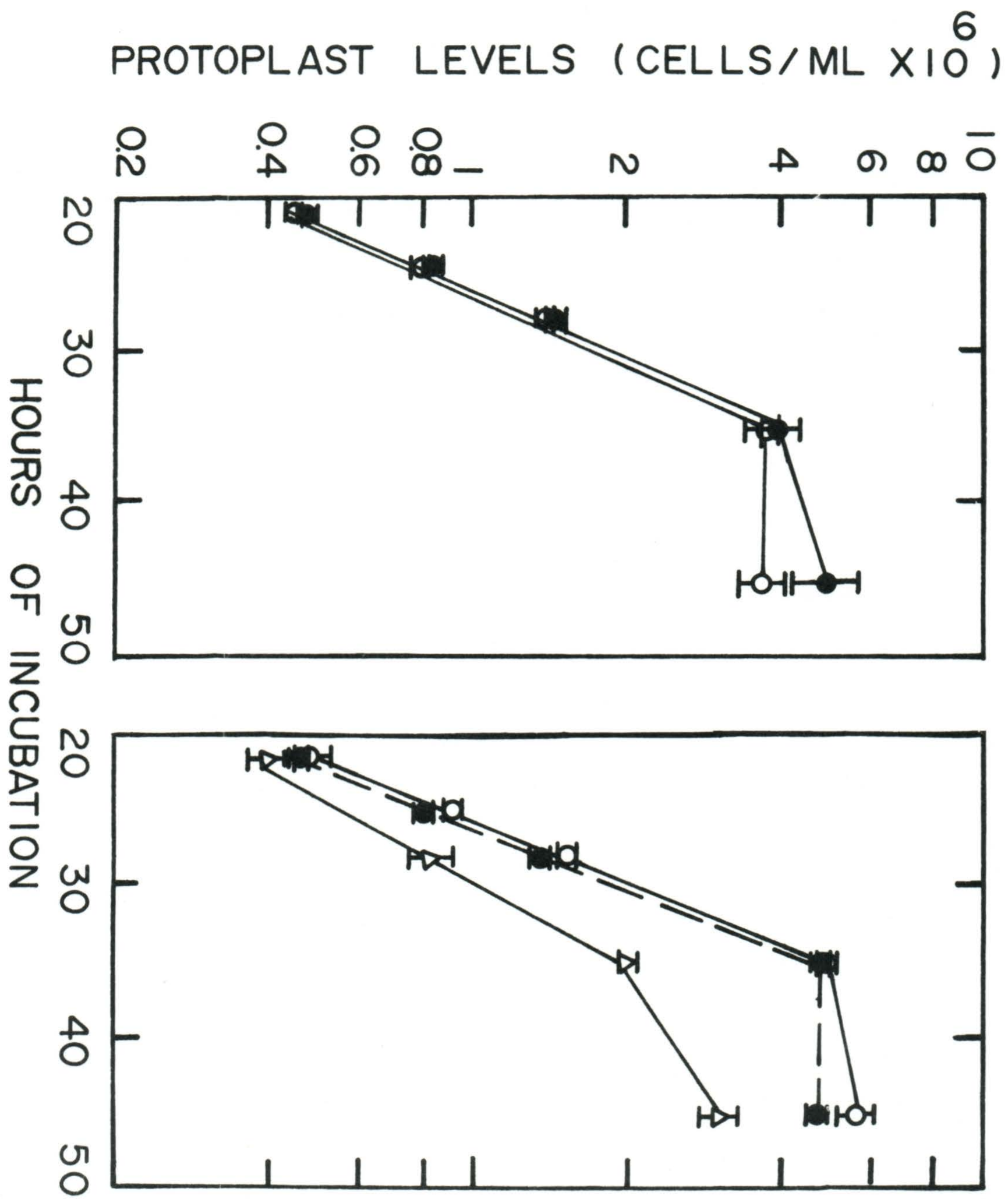
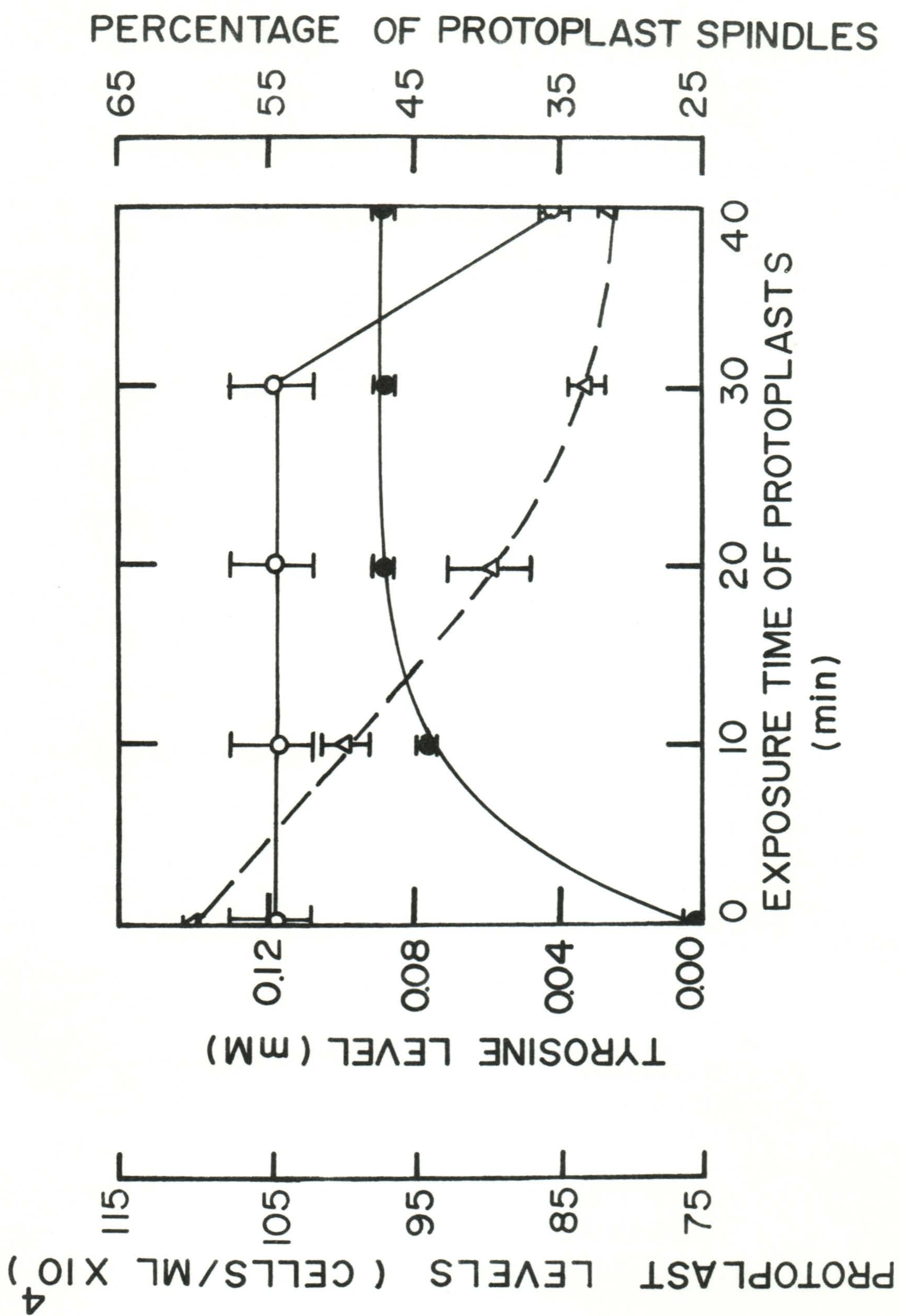


Fig. 28. The effects of 1 trypsin unit on E. egressa protoplast cell number (O), the percentage of spindle-shaped protoplasts (Δ) and tyrosine release from the protoplasts (●).





The optimum exposure times were selected as 10 and 20 min.

(c) Trypsinized protoplasts and normal hemocytes. The hemocytes did not adhere to either the control or trypsin treated protoplasts regardless of the exposure time (Plate 14, Figs. 1 and 2). The hemocytes and protoplasts behaved as described in section II. B. i. a. and ii. a.. Occasionally spherical hyphal body initials lacking discernible cell walls were also detected in trypsinized samples. These cells were also free of adhering hemocytes (Plate 14, Fig. 3).

(d) Trypsinized hemocytes and spores of *Ab-sidia repens*. Trypsinized hemocytes contained the same level of spores of *A. repens* per granular cell as the control hemocytes (Table XVII). The percentages of granular cells with spores were the same for both control and trypsinized hemocytes. The level of released tyrosine from trypsinized hemocytes was greater than that of the control cells (Table XVII) confirming trypsin-hemocyte interaction.

## 2. Papain study.

(a) Papain assay. In the initial assay papain activity was 0.032 units/ug (Fig. 29). Because of



## Plate 14

- Fig. 1. Protoplasts of Entomophthora egressa isolate 521 not exposed to trypsin revealing the absence of adhering spruce budworm hemocytes. Phase contrast. In vitro. MccT. X1200
- Fig. 2. Protoplasts of Entomophthora egressa isolate 521 after exposure to trypsin revealing the absence of spruce budworm hemocytes. Phase contrast. In vitro. MccT. X1200.
- Fig. 3. Spherical hyphal body initial (arrow) of Entomophthora egressa isolate 521 after exposure to trypsin revealing the absence of adhering spruce budworm hemocytes. Phase contrast. In vitro. MccT. X1200.
- Fig. 4. Granular cell (arrow) adhering to papain-treated protoplast of Entomophthora egressa isolate 521. Phase contrast. In vitro. MccT. X1200.

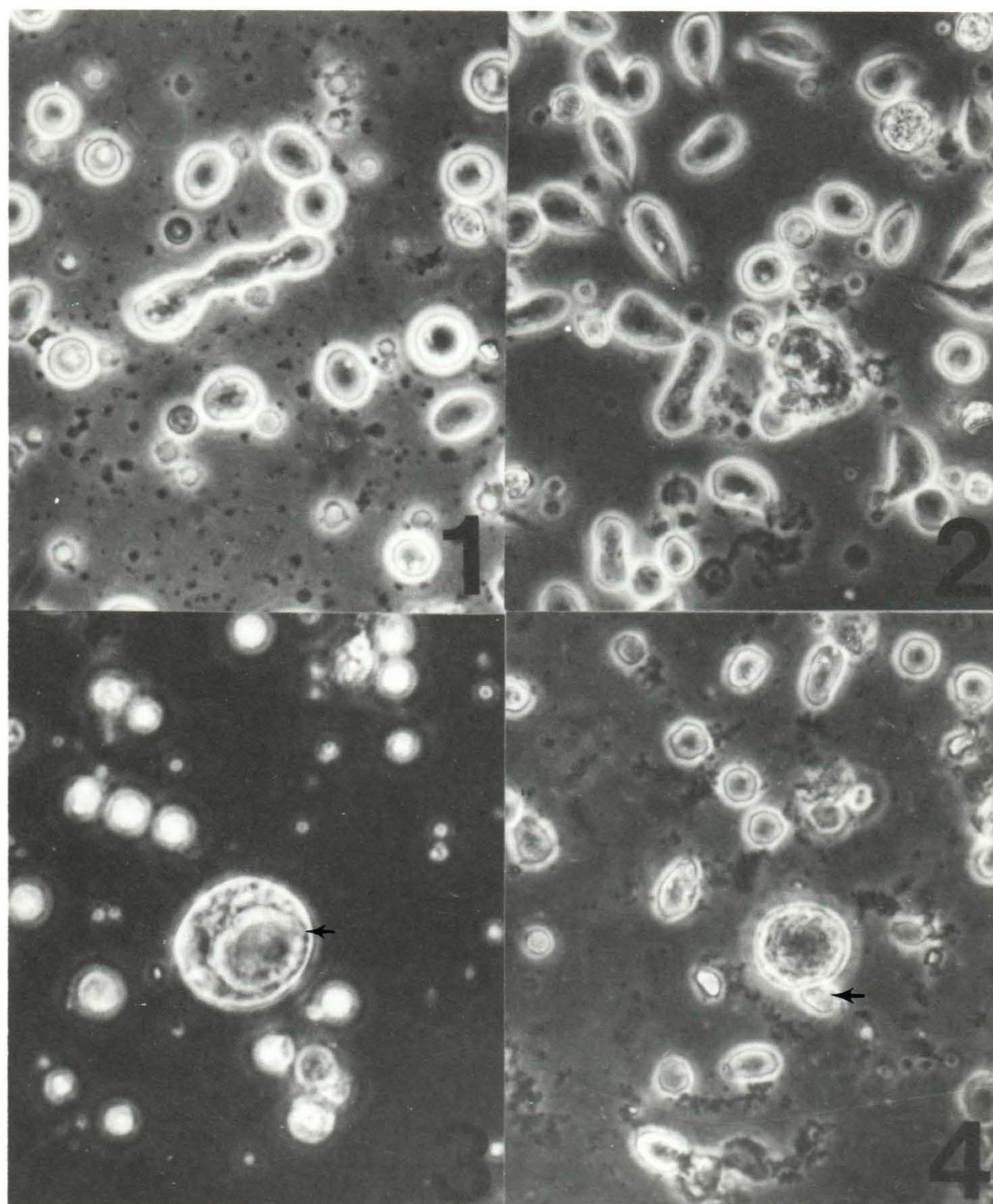




Table XVII  
Effects of trypsin on the interaction  
of spruce budworm granular cells with  
the spores of Absidia repens<sup>a</sup>

Treatment	Spores/ granular cell	% granular cells with spores	Tyrosine level in supernatant ( moles/10ml)
Trypsin	2.60 $\pm$ 0.23	98.7 $\pm$ 2.1 <sup>b</sup>	0.230 $\pm$ 0.002
Control	2.48 $\pm$ 0.31 (t=0.278, P>0.8)	100.0 $\pm$ 0.0 (t=0.619, P>0.5)	0.133 $\pm$ 0.007 (t=13.472, P<0.001)

<sup>a</sup>sample size, n=10 for control and trypsinized hemocytes

<sup>b</sup>150 granular cells were examined per sample

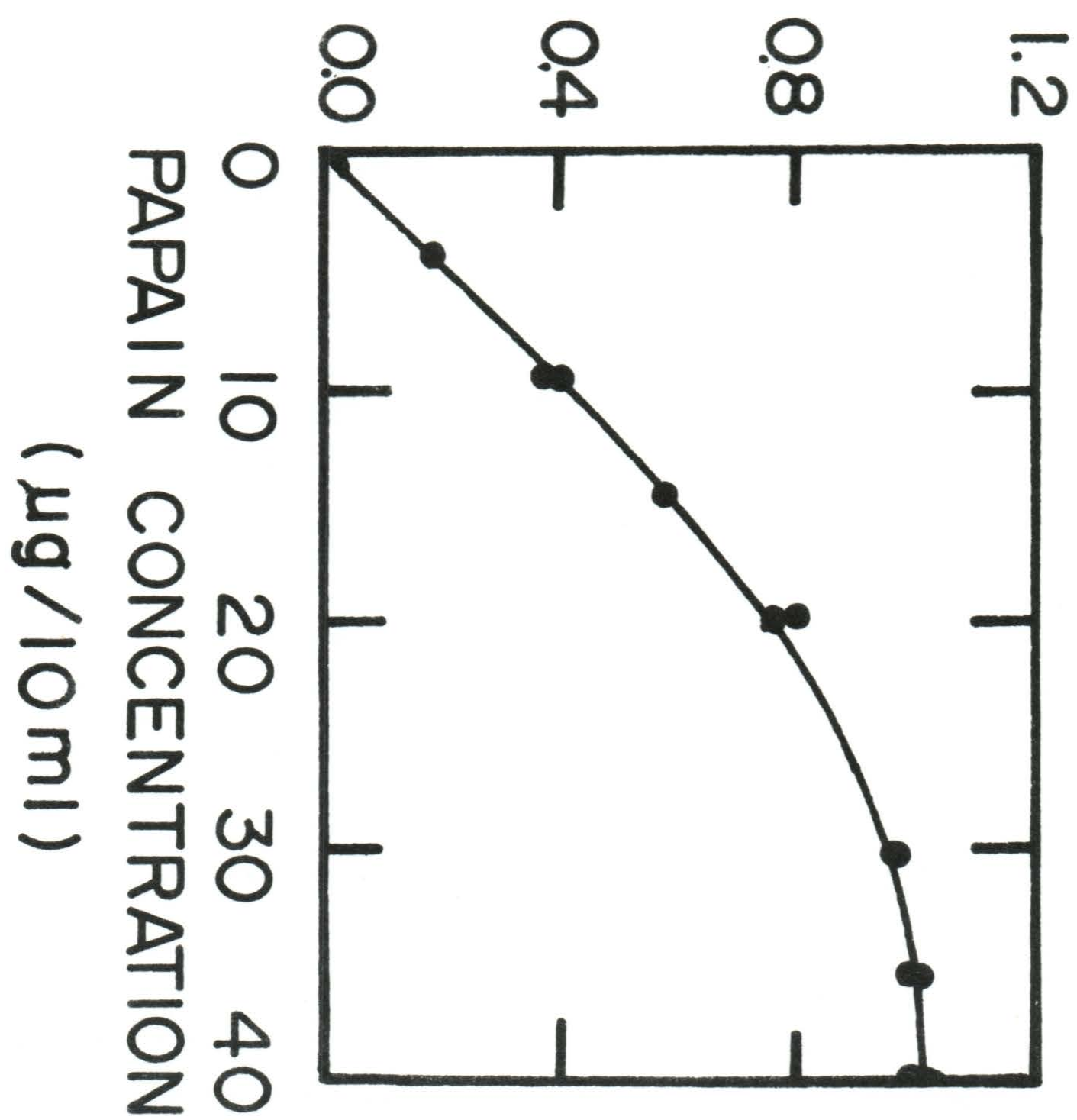
(Note: Only sixth instar female larvae used)

Fig. 29. Absorption of protein hydrolysates exposed to  
selected levels of papain.



# HYDROLYSATE ABSORPTION

( 280 nm )



the problem with absorption at 280nm for protein hydrolysates and sucrose, the hydrolysates in the sucrose based substrate medium were separated from sucrose (see section VII. B. 1.). If sucrose inhibited or stimulated papain, changes in the elution profiles would be expected. Sucrose eluted with the 219ml fraction (217-223ml) (Fig. 30). The elution of the sucrose-based and sucrose-free papain assays were similar in overall profile but differed in the magnitude of the major absorption peaks (Fig. 30). The 7ml and 14ml fractions of the sucrose-based assay had lower absorption values than the sucrose-free papain assay (Table XVIII). The total area under the curve for either fraction in the sucrose-based assay was less than the sucrose-free assay (Table XVIII). On this basis the sucrose would appear to stimulate papain activity because these fractions which contained large protein fragments diminished in concentration. The filter sterilized papain enzyme solution revealed the same elution profile as the nonfiltered sucrose-based assay. There was no significant difference between the two assays, therefore, filtering the enzyme preparation did not affect enzyme activity allowing the effects of papain on the protoplasts to be studied aseptically.

(b) Protoplast viability. Protoplasts exposed to the papain activators (cysteine and EDTA), activator plus TCA and papain and activated papain grew at the



Fig. 30. Elution profiles from a Sephadex G-10 column of sucrose and casein hydrolysates based on 1 degradation of casein by papain with and without sucrose.

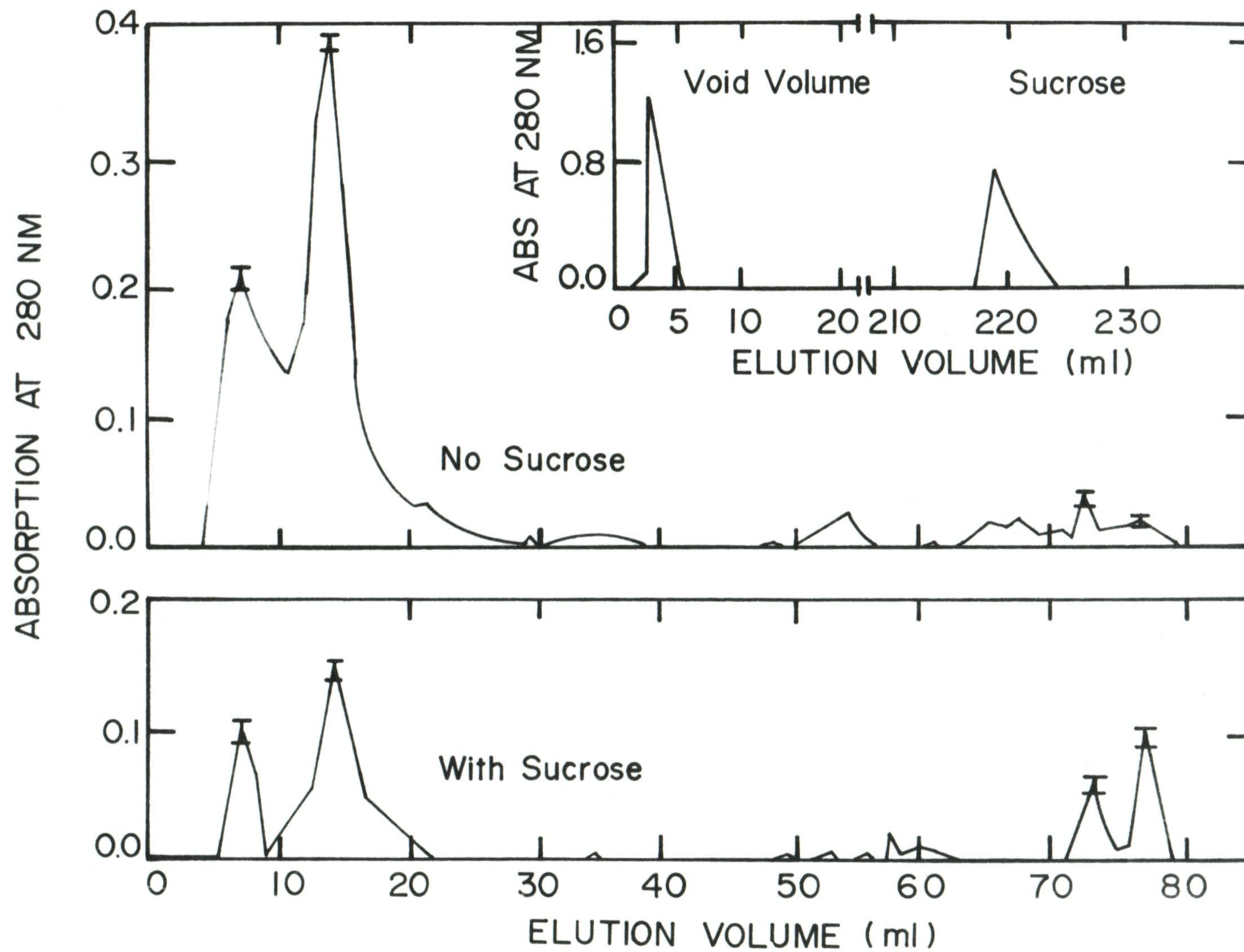




Table XVIII  
Effects of sucrose on papain activity  
using the 7 and 14ml elution fractions

Fraction	Sucrose	Abs <sup>a</sup> <sub>280nm</sub>	t-value (P-value)	Area of elution peak (cm <sup>2</sup> x10 <sup>-2</sup> )	t-value (P-value)
7ml	- <sup>b</sup>	0.217 ± 0.014 (n=2) <sup>c</sup>		186.90 ± 0.42	
7ml	+	0.113 ± 0.006 (n=3)	6.842 (P<0.01)	156.06 ± 0.22	16.264 (P<0.001)
14ml	-	0.394 ± 0.002 (n=2)		920.58 ± 2.06	
14ml	+	0.144 ± 0.009 (n=3)	27.174 (P<0.001)	285.61 ± 1.87	223.251 (P<0.001)

<sup>a</sup>Maximum peak absorption

<sup>b</sup>- No sucrose

+ With sucrose

<sup>c</sup>Sample size

same rate as the nontreated protoplast controls i.e. 5h/generation (Figs. 31 and 32).

Protoplasts of isolate 458 were also exposed to the activators and enzymes. Viability was based on the protoplast concentration in MGM after 48h of incubation. The presence of papain activators and inhibitors did not influence the overall protoplast yields (Table XIX). Papain caused a lower protoplast level.

(c) Papain-treated protoplasts and normal hemocytes. Protoplasts of isolate 521 treated with only papain were susceptible to attack by the granular cells. The number of granular cells/protoplast was highly variable averaging 3 with a range of 1 (Plate 14, Fig. 4) to 8 (Plate 15, Fig. 5) granular cells. All protoplasts with adhering hemocytes remained in the spherical form; whereas protoplasts exposed to the various activators remained in this form for 5min and returned to the normal protoplast shape within 15min of centrifugation. Papain-treated protoplasts not exposed to hemocytes also returned to normal within 15min postcentrifugation. Generally,  $95\% \pm 2\%$  of the protoplasts were associated with hemocytes.

Isolate 458 protoplasts were sensitive to the activators inhibitors (TCA) as exhibited by the adhesion of



Fig. 31. Growth of protoplasts of Entomophthora egressa isolate 521 after exposure to papain activators (0) and activators, TCA and papain (●).

Fig. 32. Growth of protoplasts of Entomophthora egressa after exposure to activated papain (▲) and as non-treated control protoplasts (Δ).

PROTOPLAST LEVELS ( CELLS / ML X 10<sup>5</sup> )

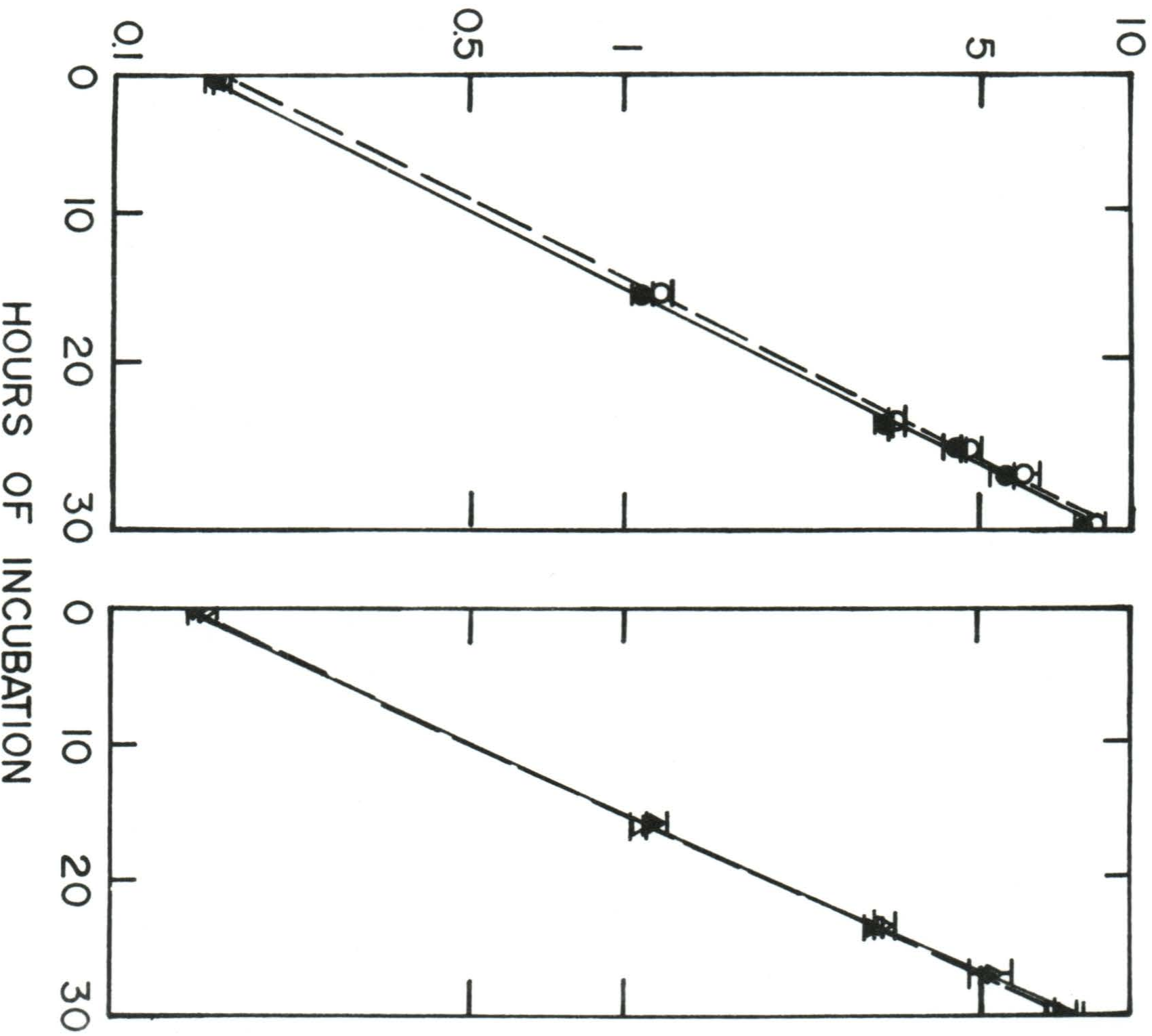




Table XIX  
Effects of papain activators, activators  
and inhibitors and papain on the viability  
of protoplasts of isolate 458

Treatment	Protoplast level (cells/ml x 10 <sup>2</sup> )	t-value <sup>a</sup> (P-value)
No activators	2.1 ± 0.2	
Activators <sup>b</sup>	1.7 ± 0.1	1.789 (P>0.1)
Activators + TCA + papain	1.5 ± 0.2	1.342 (P>0.2)
Activated papain	1.1 ± 0.1	4.472 (P<0.001)

<sup>a</sup>t-test between no-activators and the activator  
containing solutions

<sup>b</sup>cysteine (50mM) plus ethylenediaminetetraacetic  
acid (20mM) at pH8.0

## Plate 15

Fig. 5. Granular cell rosette around a papain-treated protoplast (arrow) of Entomophthora egressa isolate 521. Phase contrast. In vitro. MccT. X1200.



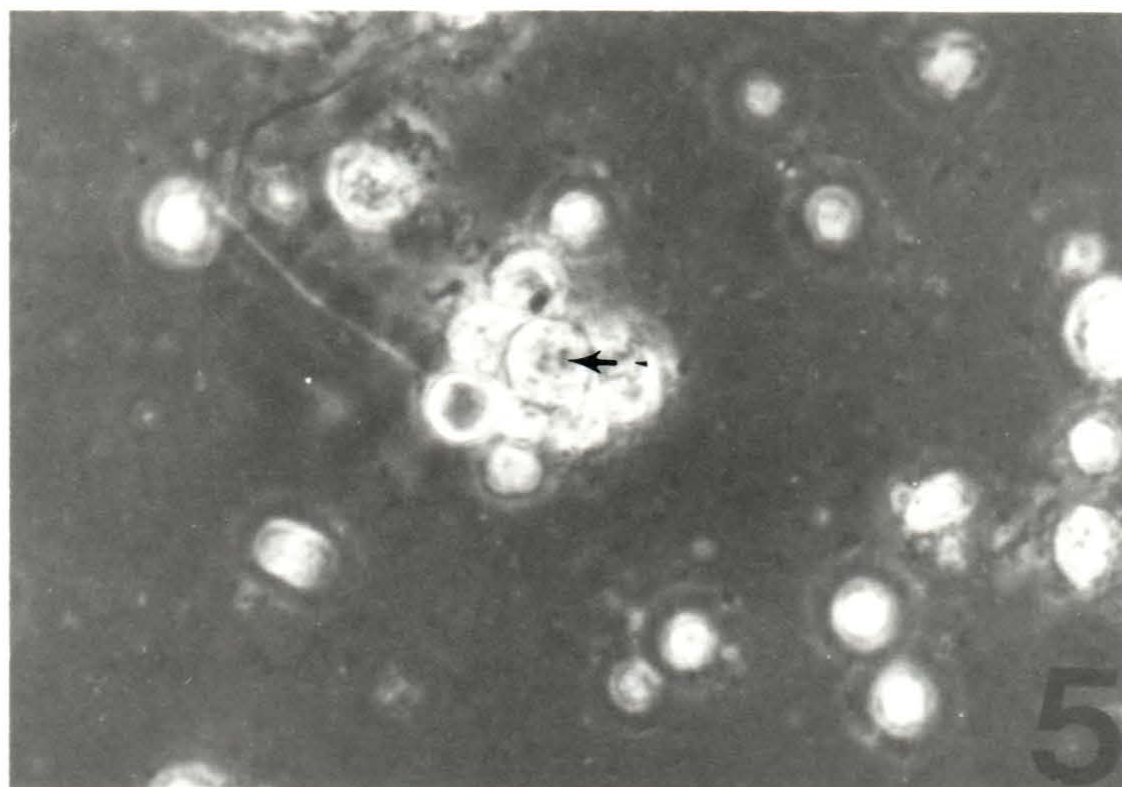


Table XX  
Effects of papain activators, inhibitors and  
papain on protoplast (isolate 458) susceptibility  
to attack by spruce budworm granular cells

Treatment	Hemocytes/ protoplast	t-value (P-value)	% protoplasts with hemocytes	t-value (P-value)
No activators	0 $\pm$ 0 (n=50)		0 $\pm$ 0	
Activators	1.3 $\pm$ 0.2 (n=50)		7.8 $\pm$ 2.2	
Activators + TCA + papain	1.7 $\pm$ 0.1 (n=50)	1.789 <sup>a</sup> (P>0.1)	10.2 $\pm$ 1.2	1.029 (P>0.2)
Activated papain	2.9 $\pm$ 0.1 (n=50)	8.486 <sup>b</sup> (P<0.001)	91.1 $\pm$ 2.0	34.691 (P<0.001)

<sup>a</sup>Activators compared to Activators + TCA + papain

<sup>b</sup>Activators + TCA + papain compared to Activated papain

(Note: Only sixth instar female larvae used)



Table XXI  
Effects of papain on the adhesion of spores of  
Absidia repens to spruce budworm granular cells

Treatment	Spores/ granular cell	t-value (P-value)	% granular cells with spores	t-value (P-value)
Sucrose	3.2 $\pm$ 0.1		98.3 $\pm$ 1.2	
Activators	3.0 $\pm$ 0.2	t= 0.894 <sup>a</sup> (P > 0.5)	96.1 $\pm$ 1.7	t= 1.057 (P > 0.2)
Activators + TCA + papain	3.1 $\pm$ 0.3	t= 0.316 (P > 0.75)	97.3 $\pm$ 2.3	t= 0.384 (P > 0.75)
Activated papain	0.9 $\pm$ 0.1	t=16.264 (P < 0.001)	2.7 $\pm$ 0.5	t=72.267 (P < 0.001)

<sup>a</sup>t-test comparing the results of the sucrose control with every other test medium

(Note: Only sixth instar female larvae used)

B. Discussion. The fact that protoplasts of isolate 521 were harmed by 30 and 40min exposure to trypsin and yet the surviving cells grew at rates exceeding the control protoplasts or protoplasts exposed to trypsin for 10 and 20 min indicates that the more vigorous protoplasts may have been resistant to trypsin.

The failure of trypsinized protoplasts to favor granular cell adhesion may be indicative of several possibilities:

(i) the sites of molecular mimicry are not connected to proteins since proteolytic activity, based on tyrosine release was detected.

(ii) the mimic sites are connected to trypsin insensitive proteins. Trypsin insensitivity might be attributed to a paucity of lysine and/or arginine residues in the proteins or to proteins with such residues which by virtue of the spatial orientation of the protein make the tyrosine sites inaccessible to trypsin.

The broad spectrum activity of papain would ensure a more nonselective cleavage of protoplast surface proteins.



The protoplast growth rates and granular cell responses to protoplasts of isolate 521 exposed to papain activators and inhibitors confirmed that the protoplasts were not harmed. Protoplasts of isolate 458 were more sensitive to the activators. The low level of damaged cells would suggest that the samples of isolate 458 protoplasts were more heterogeneous in character than those of isolate 521.

The adhesion of granular cells to protoplasts of both isolates exposed to papain establishes the presence of recognition groups are proteins or connected to proteins at the protoplast cell membrane. That recognition of foreignness by insect hemocytes is a surface phenomenon has been reported by Nappi (1973, 1974), Vinson (1977) and Ratcliffe and Rowley (1979). The concept of molecular mimicry, which is strongly supported by the enzyme-based studies of the present work in addition to the results of section II. B., has been evoked to explain the survival of Trypanosoma brucei Plimmer and Bradford in Blabera fusca L. (Gobert et al. 1977), and the cestode Hymenolepis diminuta Rudolphi in T. molitor (Lackie 1976).

The spherical form of protoplasts of isolate 458 and 521 with adhering hemocytes is similar to the results with T. molitor (section II. B. 1. ii. b) and is indicative of stress.



The adhesion of spores of A. repens to trypsinized hemocytes to the same magnitude as those of the control hemocytes may stem from several sources:

(i) trypsin may have triggered the granular cells to become adhesive

(ii) trypsin may not have cleaved the putative recognition sites on the granular cells.

Scott (1971) reported that trypsin reduced the uptake of sheep erythrocytes by the hemocytes of P. americana L. The reduced interaction of hemocytes exposed to papain with A. repens spores substantiates Scott's (1971) proposal that hemocytes contained protein receptor sites.

In view of the absence of humoral opsonins (factors enhancing phagocytosis) a feature common to most insects (Anderson 1976) and the papain effects, it would appear that the recognition sites exist on the hemocyte cell membrane. Their activity may be suppressed or enhanced by carbohydrates. Parish (1977) has proposed a model for cytophilic recognition factors based on glycosyltransferases. The present data and the extending conjecture complement the recognition mechanisms and responses to non-self agents in P. brassicae (Anderson 1976, Ratcliffe and Rowley 1979) and G. mellonella (Rowley and Ratcliffe 1976).



VI. Hemolymph volume and serum protein analysis of selected stages of male and female larval spruce budworm.

Prior to determining the effects of protoplasts on larval serum it was necessary to determine the hemolymph volume, total protein content and electrophoretic protein profile of selected stages of male and female spruce budworm larvae. The object was to determine if differences between sexes or within a stage for a given sex could be detected which could restrict the usage of larvae in the interaction studies.

A. Results.

1. Hemolymph volume. Amaranth dye stained the muscularis around the mesenteron and the Malpighian tubules. The hemolymph levels declined logarithmically (Fig. 33). Based on the extrapolation of dye concentration to "0"min, the spruce budworm larvae reduced the amaranth concentration in the hemolymph by 23.5% 5min post injection. The hemolymph volumes for individual larvae were corrected for dye uptake.

The correlations of hemolymph volume with either larval mass or age were not significant for both sexes (Table XXII). Consideration of hemolymph volume with larval age and mass

Fig. 33. Decline in amaranth dye concentration in larval hemolymph over 15min post injection. Broken line represents extrapolation to "0"min.



# AMARANTH CONCENTRATION

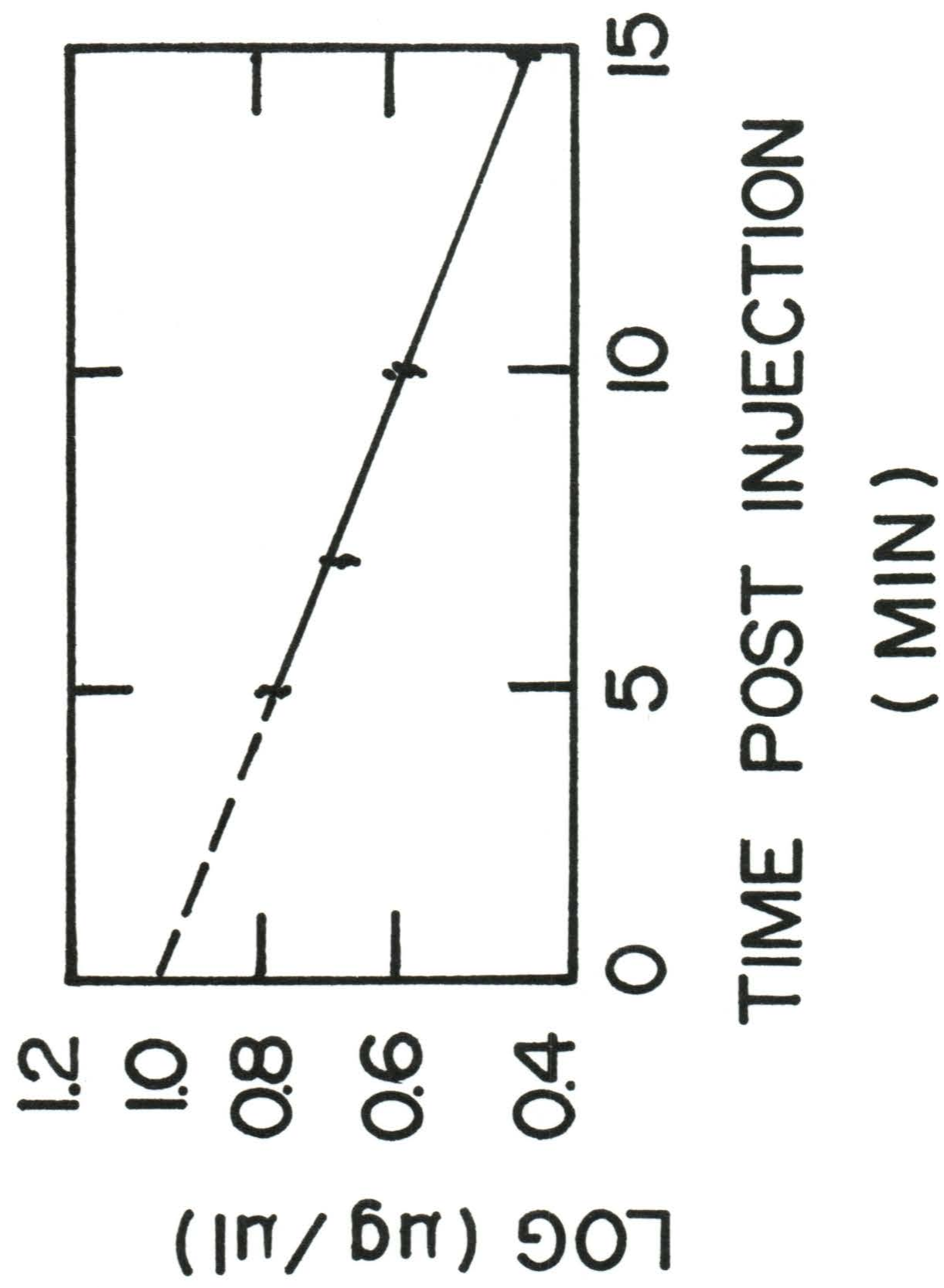


Table XXII

Correlation coefficients between hemolymph volume,  
larval mass and age of male and female spruce budworm

Variables	Sex	
	male	female
volume:larval mass	0.235 (P>0.5)	0.321 (P>0.5)
volume:larval age	0.178 (P>0.5)	0.153 (P>0.5)
volume/mass:larval age	0.328 (P>0.4)	0.381 (P>0.4)
volume:mass:larval age	0.978 (P<0.001)	0.980 (P<0.001)



produced highly significant results (Table XXII).

The regression equations used for hemolymph volume estimates were as follows:

(i) For the female larvae:  $Y = 6.14481 - 0.0434X_1 + 0.190X_2$ ,  
 where Y is hmeolymph volume;  
 $X_1$  is larval mass in mg and  
 $X_2$  is larval age in days

(ii) for the male larvae:  $Y = 5.987 - 0.0730X_1 + 0.1031X_2$ .

From the original data and above equations it was determined that female larvae contained more hemolymph than did male larvae of equal mass and age. The hemolymph volume:mass ratios were the same for all stages of a given sex and between sexes for a given stage of development (Table XXIII).

2. Total serum protein levels. The total protein levels in the female larval serum increased from the third instar to the fifth instar by a factor of 5.4 and remained constant throughout the fifth and sixth instars (Fig. 34). In the male larvae the protein content increased gradually from the third instar until the early sixth instar. This was followed by a sudden 2.3 fold increase in protein level 12 days later in the late sixth instar (Fig. 34).

Table XXIII

Hemolymph volume to larval mass ratios for male and female spruce budworm in selected stages of development

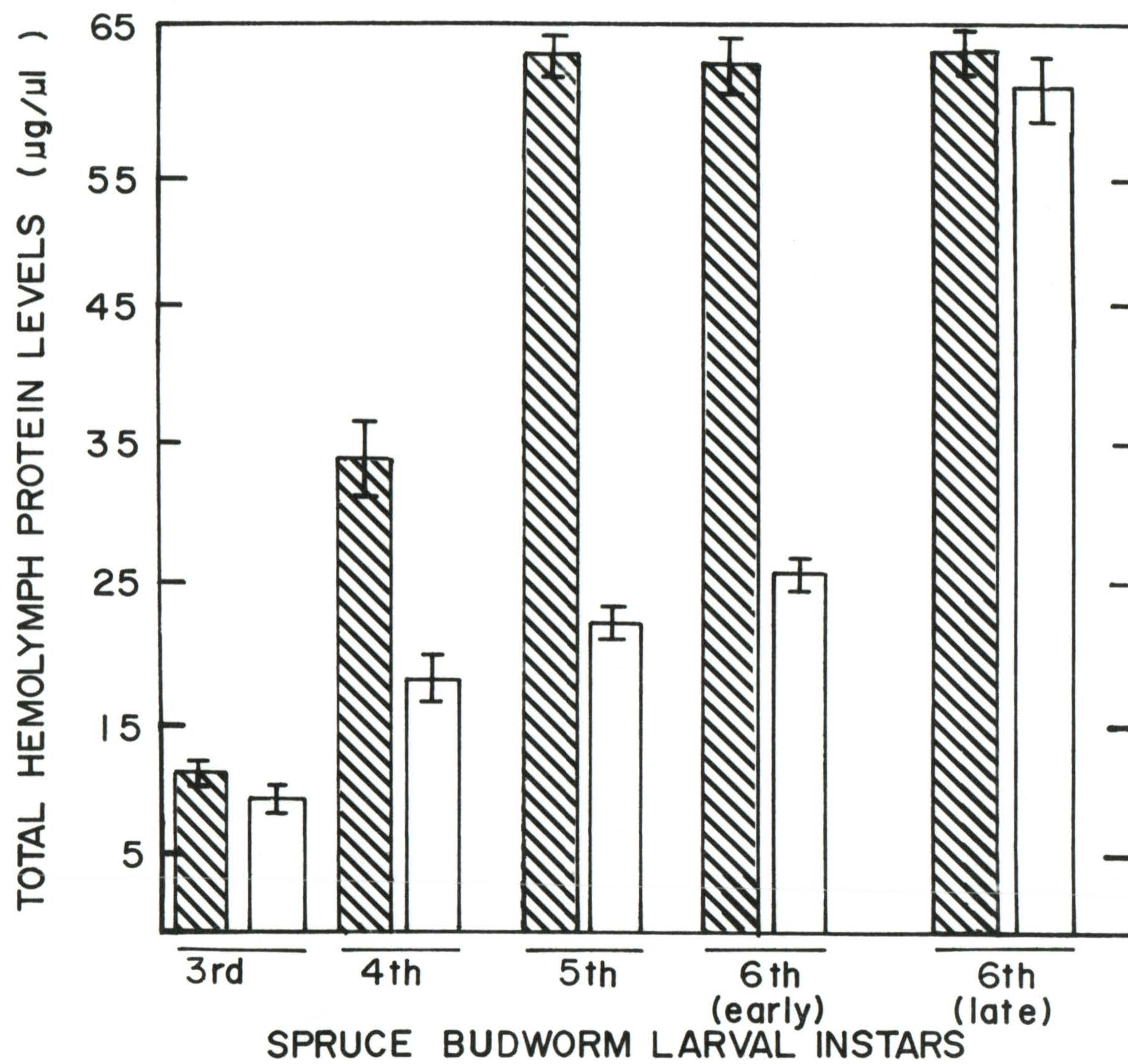
Instar	Sex		
	male	female	
Third	33.0 $\pm$ 3.5	32.7 $\pm$ 1.9	t=0.075 (P>0.9) <sup>a</sup>
Fourth	30.2 $\pm$ 2.1	28.9 $\pm$ 2.6	t=0.389 (P>0.7)
Fifth	24.8 $\pm$ 3.1	30.2 $\pm$ 4.1	t=1.051 (P>0.1)
Sixth	33.4 $\pm$ 5.1	25.1 $\pm$ 2.2	t=0.306 (P>0.7)
	F=0.871 (P>0.5) <sup>b</sup>	F=0.723 (P>0.5)	

<sup>a</sup>t-value between sexes for a given larval instar

<sup>b</sup>F-value for a given sex



Fig. 34. Changes in the levels of total hemolymph protein during the development of female (N) and Male (I) spruce budworm larvae.





With the exception of the third and late sixth instars the female larvae had greater total serum protein levels than the males (Fig. 34).

3. Electrophoresis of larval serum. The supposition that identically located bands on polyacrylamide gels necessarily represent the same proteins or that proteins cannot be the same because they differ in electrophoretic mobility ( $R_m$  values) is not strictly true (Whitmore and Gilbert 1974). In presenting the current results it will be assumed that protein bands with identical  $R_m$  values for a given species at different stages of development are at least related proteins as proposed by Whitmore and Gilbert (1974). The number of protein bands observed on the polyacrylamide gels was greatest in fresh serum from female sixth instar larvae and least in frozen serum (Plate 16, Fig. 1; Fig. 35).

Comparing the  $R_m$ 's of the bands of the fresh female sixth instar serum with those of the fourth and fifth instars revealed that the fourth instar had 5 detectable bands and the fifth instar had 10 bands in common with the sixth instar (Plate 17, Fig. 1; Fig. 35). The frozen serum had 4 bands in common with the nonfrozen serum. Many of the common bands differed between developmental stages in their width even though the same level of total protein was applied to each gel.

## Plate 16

Fig. 1. Electropherograms of the serum of selected stages of male and female spruce budworm larvae. M6 - male, 6th instar; F6 - female, 6th instar; F5 - female, 5th instar; F4 - female, 4th instar; F6f - female, 6th instar, frozen.



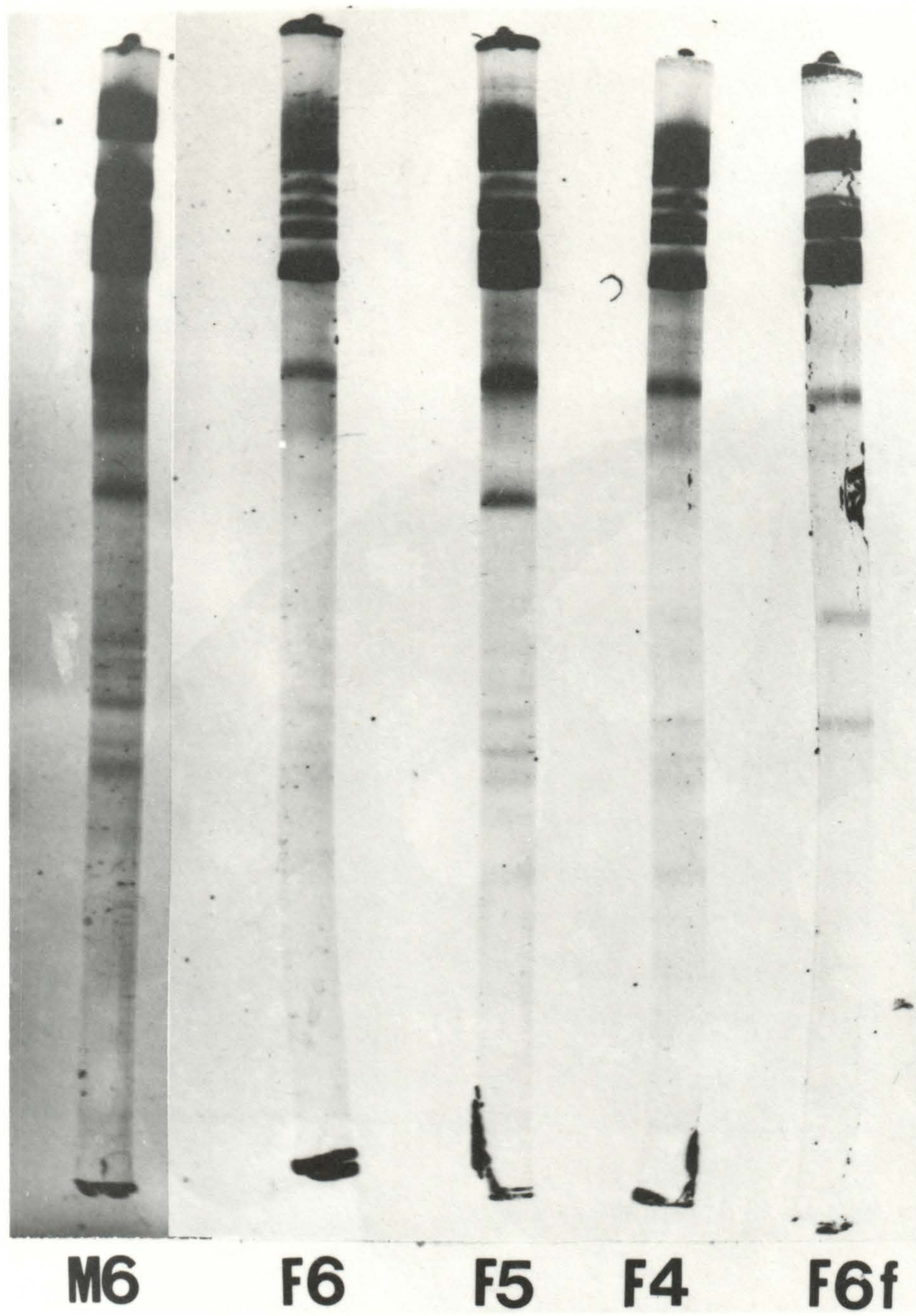
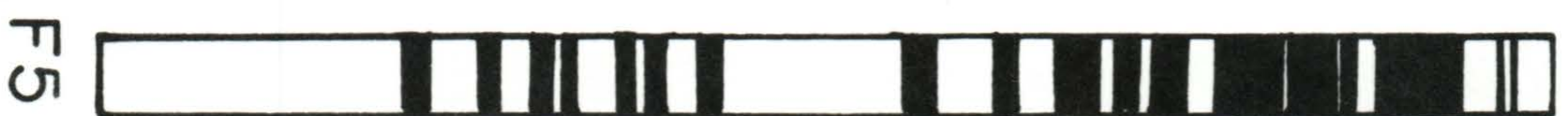
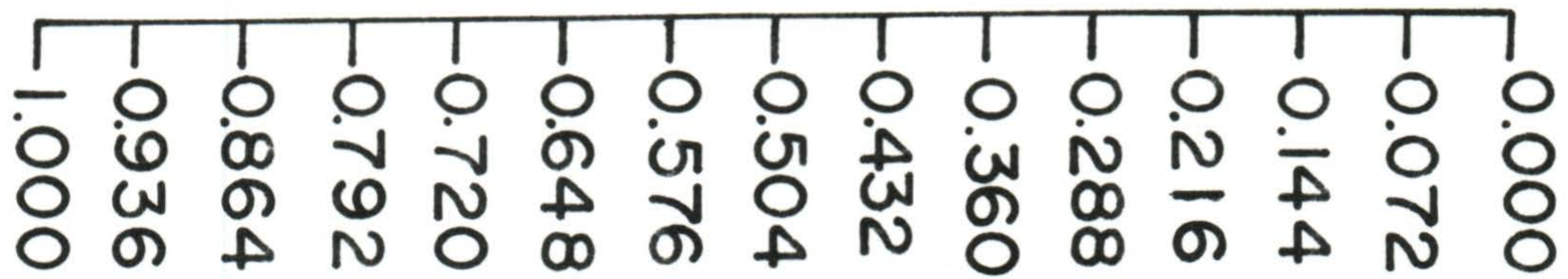


Fig. 35. Tracing of Plate 16, Fig. 1. Refer to Plate 16,  
Fig. 1 for coding.



RM SCALE



There were 4 major bands in all stages examined. With increasing larval development the total protein concentration increased, the number of protein bands increased and the width of many of the bands increased up to the fifth instar. During the sixth instar the band widths decreased for many of the protein bands.

Serum from the sixth instar males contained three bands common to the female larvae (Plate 16, Fig. 1; Fig. 35). Many of the slowly migrating proteins, Rm 0-0.250 range, of the males, because of the width, overlapped with the more narrow bands of the females. The sixth instar females revealed a yellow-blue chromoprotein band at Rm 0.758<sup>±</sup> 0.013 which was absent in the males.



B. Discussion. The uptake of amaranth dye by the Malpighian tubules of the spruce budworm larvae is similar to the amaranth dye removal by P. americana (Yeager and Munson 1950), Schistocerca gregaria Forsk. (Lee 1961) and Evans blue from Calliphora vicina Meig. (Levenbook 1979). Dye uptake by the hemocytoblastic organs by C. vicina has been reported by Levenbook (1979). The uptake of dye by the gut muscularis appears to be unique.

The hemolymph volume of spruce budworm was related to larval age, mass and sex. Gagen and Ratcliffe (1976) reported that the hemolymph volumes of P. brassicae and G. mellonella were highly correlated with larval mass. The volume of insect hemolymph has been reported to vary with the species of insect, stage, age within a stage, diet, insect activity and method of determination (Lee 1961, Lough-ton and Robe 1969, Jones 1977, Levenbook 1979).

Both the male and female spruce budworm larvae maintained equal and constant hemolymph volume per mass percentages (25-35%) throughout development. However, because the mass of the larvae increased with age, the absolute hemolymph volumes also increased. A similar pattern has been reported for S. gregaria nymphs (Lee 1961), Phormia regina Meig. (Chen and Levenbook 1966) and the early larval stages of C. vicina (Levenbook 1979). The percentage of hemolymph



volume of larval mass of G. mellonella has been found to range from 30-33% (Gagen and Ratcliffe 1976). Gagen and Ratcliffe (1976) and Brugnon and Le Berre (1976) have reported the same values for P. brassicae.

The existence of differences in absolute hemolymph volumes between male and female spruce budworm larvae has counterparts in adult S. gregaria (Lee 1961), Musca domestica L. (Bodnaryk and Morrison 1966), adult Locusta migratoria migrataroides L. (Loughton and Tobe 1969), larvae and pupae of B. mori (Horie et al. 1971) and sixth instar P. brassicae (Turunen and Junnilskalla 1974). In all cases the females contained more hemolymph than the males.

The abrupt increase in the serum protein content of the male spruce budworm during the late last instar is similar to reports for B. mori (Wyatt et al. 1956), Antheraea pernyi Guer. and Hyalophora cecropia L. (see Wyatt 1961), Samia cynthia Drury (Laufer 1960), P. brassicae (Van der Geest 1968) and E. kühniella (Cölln 1973). The increase in female serum protein during the middle instars is analogous to that reported for many holometabolous insects in general (Thomson 1975).

The female spruce budworm, depending on the stage, usually contained more serum protein than the corresponding



males. Male larvae of H. cecropia, Hyalophora gloverii L. and Callosamia promethea Jefferson contained less hemolymph protein than the female larvae (Whitmore and Gilbert 1974).

The quantitative and qualitative changes in the electrophoretic protein profiles of the female spruce budworm larvae have parallels in the larvae of Philosamia (= Samia) cynthia Drury, H. gloverii (Whitmore and Gilbert 1974), Manduca sexta Johannson (Greene and Dahlman 1973) and Dendrolimus pini L. (Luzev and Belozarov 1977).

The decrease in the width of several serum protein bands and the absence of several as the spruce budworm developed may represent the selective uptake of hemolymph proteins by the late instar fat body. Sequestering of hemolymph proteins by larval fat body with the formation of proteinaceous spheres has been reported for P. brassicae (Chippendale and Kilby 1969), G. mellonella (Collins and Downey 1970), H. cecropia (Patel 1971) and E. kuhniella (Colln 1973).

The electropherograms of the serum male and female spruce budworm larvae showed differences between the sexes. Qualitative and quantitative differences in electropherograms of male and female H. gloverii have been documented by Whitmore and Gilbert (1974). The presence of chromoproteins in the female budworm larvae is not unique. These proteins have



been found in a number of lepidopterous insects including Choristoneura viridis Freeman and Choristoneura occidentalis Freeman (Schmidt and Young 1971).

From the present study it was noted that freezing hemolymph altered the resolution of the proteins and influenced band number. Wyatt and Pan (1978) reported that freezing reduced the resolution of insect lipoproteins.

It was apparent that the stage of larval development, sex and method of hemolymph storage influenced the electropherograms. Because of the larger hemolymph volume and greater protein level of the females compared to the males, only sixth instar females were used in the following experiments.



VII. Interaction between spruce budworm serum and protoplasts of *Entomophthora egressa*.

A. Results.

1. Viable protoplasts added to whole hemolymph.

The level of total protein in the hemolymph controls was greater than in hemolymph containing protoplasts (Table XXIV). The protein carryover with the protoplast inoculum was believed to be negligible in view of the low protein level in the protoplast control samples (Table XXIV).

The electropherograms of the spruce budworm larvae did not reveal identical bands for most of the minor protein bands and differed in the amounts of major bands for different collections of larvae. For a given collection the results were always identical. To offset this problem separate control samples were run for each collection.

Because of problems in photographing the minor bands on the polyacrylamide gels, gel tracings showing these bands were placed next to the gel in question for the following experiment only. Thereafter, only  $R_m$  values will be presented in tabular form.

The two bands with  $R_m$  values of  $0.095 \pm 0.003$  and

Table XXIV  
Effects of incubating protoplasts of Entomophthora  
egressa with spruce budworm hemolymph

Variables	Total protein concentration (ug/ul)	t-value <sup>a</sup> (P-value)
Sucrose + protoplasts	0.04 ± 0.00	
Hemolymph + sucrose	5.75 ± 0.43	
Hemolymph + protoplasts+ sucrose	3.87 ± 0.62	2.492 (P<0.05)

<sup>a</sup>t-test between the total protein level in hemolymph + sucrose and hemolymph + protoplasts

(Note: Only sixth instar female larvae used)



0.634 $\pm$ 0.005 present in the control hemolymph electropherograms were absent in the hemolymph samples containing viable protoplasts (Plate 17, Fig. 1; Fig. 36).

This latter system contained the bands Rm 0.709 $\pm$ 0.011 and 0.652 $\pm$ 0.009 which were absent in the hemolymph controls. No bands were detected for the protoplast control samples. The protein bands common to both hemolymph regimes did not change in width in this or succeeding experiments.

## 2. Viable protoplasts added to larval serum.

The serum with viable protoplasts differed from the serum control and hemolymph samples in that the former lacked the band Rm 0.335-0.342 (Table XXV). The band Rm 0.782-0.794, while absent in the hemolymph electropherograms was present in both the serum with and without protoplasts. This band may be an artifact of technique.

## 3. Cold fixed and ethanol-fixed protoplasts.

Chilling at 4°C for 18h alone did not harm the protoplasts which achieved the same level of growth at 20°C as the non-chilled protoplast control (Table XXVI). Relative to the level of chilled protoplasts at 0 hours of incubation at 20°C after chilling the final yield represented an increase by a factor of 200. The ethanol treated protoplasts were killed as evident by the absence of an increase in cell number over

## Plate 17

Fig. 1 Electropherograms indicating the effects that protoplasts of Entomophthora egressa isolate 521 have on hemolymph of sixth instar female spruce budworm larvae (P) compared to control hemolymph (C).





Fig. 36. Tracing of electropherograms of Plate 17,

Fig. 1.



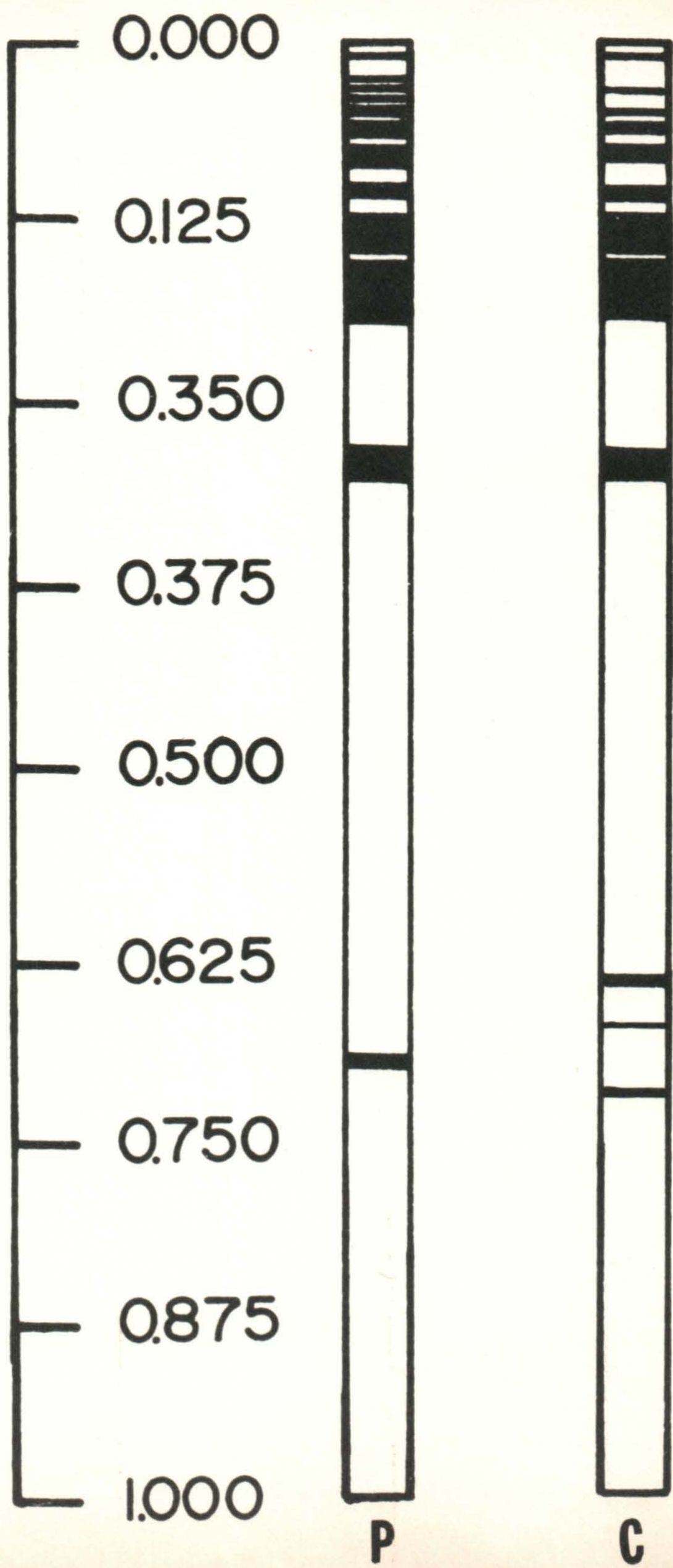




Table XXV  
Influence of viable protoplasts of  
Entomophthora egressa on hemolymph and  
serum proteins of the sixth instar female  
larvae of the spruce budworm

Rm values		
Hemolymph <sup>a</sup>	Serum	Serum control <sup>a</sup>
0.021 ± 0.004	0.030 ± 0.002	0.028 ± 0.003
0.035 ± 0.001	0.036 ± 0.002	0.036 ± 0.003
0.056 ± 0.002	0.057 ± 0.002	0.055 ± 0.003
0.085 ± 0.003	0.087 ± 0.002	0.088 ± 0.005
0.099 ± 0.002	0.103 ± 0.003	0.100 ± 0.003
0.123 ± 0.001	0.128 ± 0.006	0.127 ± 0.004
0.147 ± 0.004	0.151 ± 0.002	0.149 ± 0.003
0.231 ± 0.001	0.238 ± 0.005	0.234 ± 0.004
0.316 ± 0.000	0.317 ± 0.003	0.319 ± 0.003
0.335 ± 0.004	<sup>c</sup>	0.342 ± 0.006
0.500 ± 0.000	0.489 ± 0.15	0.491 ± 0.003
0.527 ± 0.016	0.542 ± 0.007	0.533 ± 0.008
<sup>b</sup>	0.794 ± 0.004	0.782 ± 0.006
0.802 ± 0.008	0.816 ± 0.004	0.811 ± 0.005

<sup>a</sup>Without added protoplasts

<sup>b</sup>Absent from the hemolymph samples only

<sup>c</sup>Absent in serum samples with viable protoplasts



Table XXVI  
Levels of chilled and ethanol-treated  
protoplasts after 24h incubation in MGM<sup>a</sup>

Treatment	Protoplast level (cells/mlX10)	t-value (P-value)
Control	$2.2 \times 10^3 \pm 0.1 \times 10^3$	
Chilling	$2.0 \times 10^3 \pm 0.1 \times 10^3$	$1.414^b$ ( $P > 0.15$ )
Chilling in ethanol	$1.0 \times 10^1 \pm 0.1 \times 10^1$	$198.100$ ( $P < .001$ )
All treatments at "0" hours incubation	$1.1 \times 10^1 \pm 0.1 \times 10^1$	

<sup>a</sup>Modified Grace's insect tissue culture medium

<sup>b</sup>t-value comparing Control protoplast yeilds  
to "Chilling" and "Chilling in ethanol" yields

the initial inoculum level (Table XXV,  $t=0.707$ ,  $P>0.4$ ).

Both treatments induced the protoplasts to become spherical. Each cell contained a large vacuole (Plate 18, Figs. 1 and 2). Only the chilled protoplasts returned to the normal protoplast form at 20°C. Both the cold-fixed and the ethanol-fixed protoplasts revealed an intact cell membrane as indicated by the lack of cytoplasm extruding into the medium and a sharply defined periphery based on observations at the light microscope level (Plate 18, Figs. 1 and 2). Granular cells did not adhere to the protoplasts killed by ethanol (Plate 18, Fig. 3). This enhanced the belief that the protoplast cell membrane was free from damage.

Exposure of hemolymph to either viable or nonviable protoplasts resulted in the formation of protein bands absent from the control hemolymph gels (Table XXVII). Bands present in the control groups were absent from the viable and nonviable protoplast-hemolymph samples.

4. Proteolytic activity of viable and nonviable protoplasts. The fact that nonviable protoplasts induced changes in the hemolymph electropherograms which were similar to the changes in profile caused by viable protoplasts did not rule out the possibility of an active protease on the



## Plate 18

- Fig. 1. Protoplasts of Entomophthora egressa isolate 521 chilled at 4°C for 18h. Bright field. In vitro. ML. X900.
- Fig. 2. Protoplasts of Entomophthora egressa isolate 521 chilled at 4°C for 18h in a 4% ethanol-sucrose-MES buffer solution. Phase contrast. In vitro. ML. X900.
- Fig. 3. Protoplasts (arrows) of Entomophthora egressa isolate 521 killed by ethanol exposed to hemolymph of the spruce budworm revealing the absence of granular cells (double arrows) adhering to the protoplasts. Phase contrast. In vitro. ML. X900.

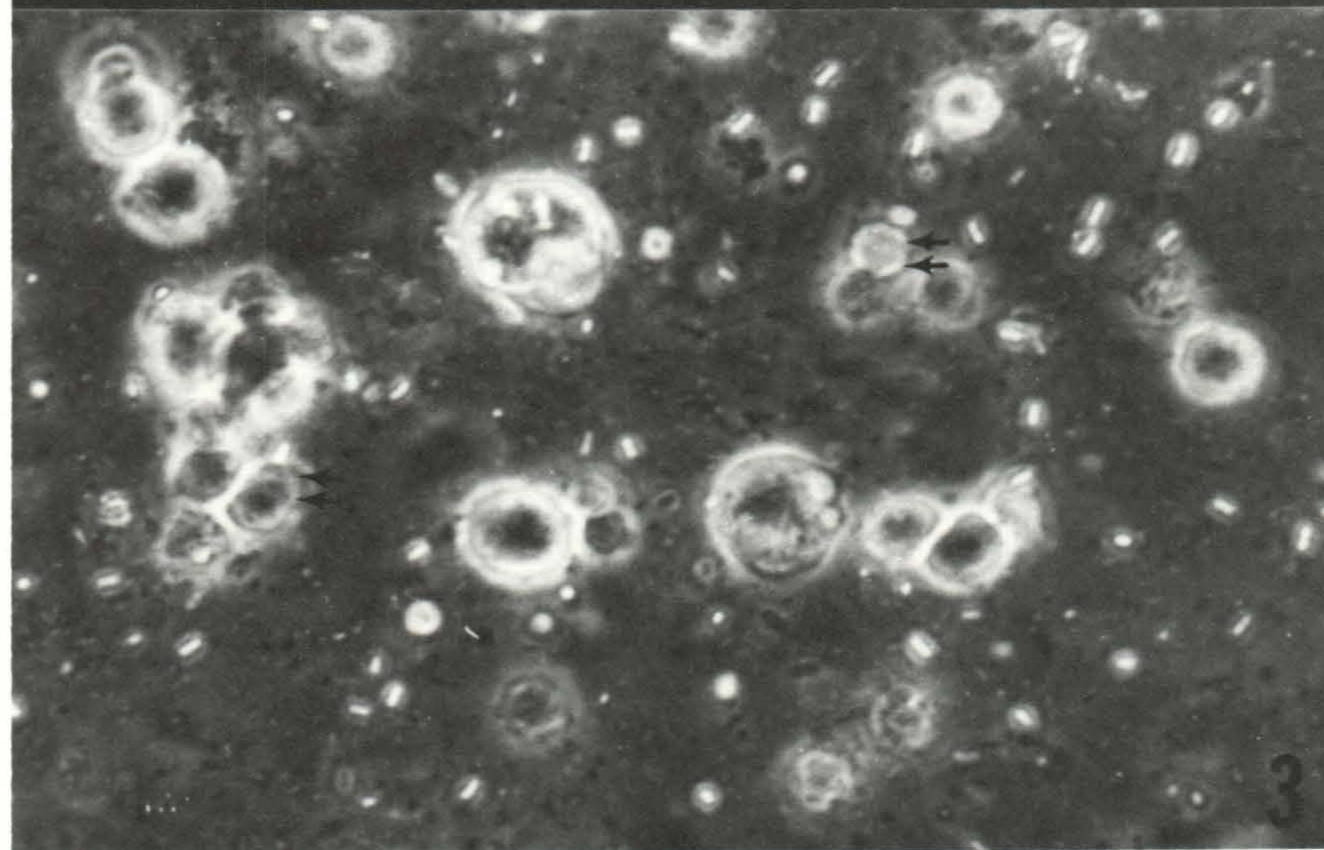
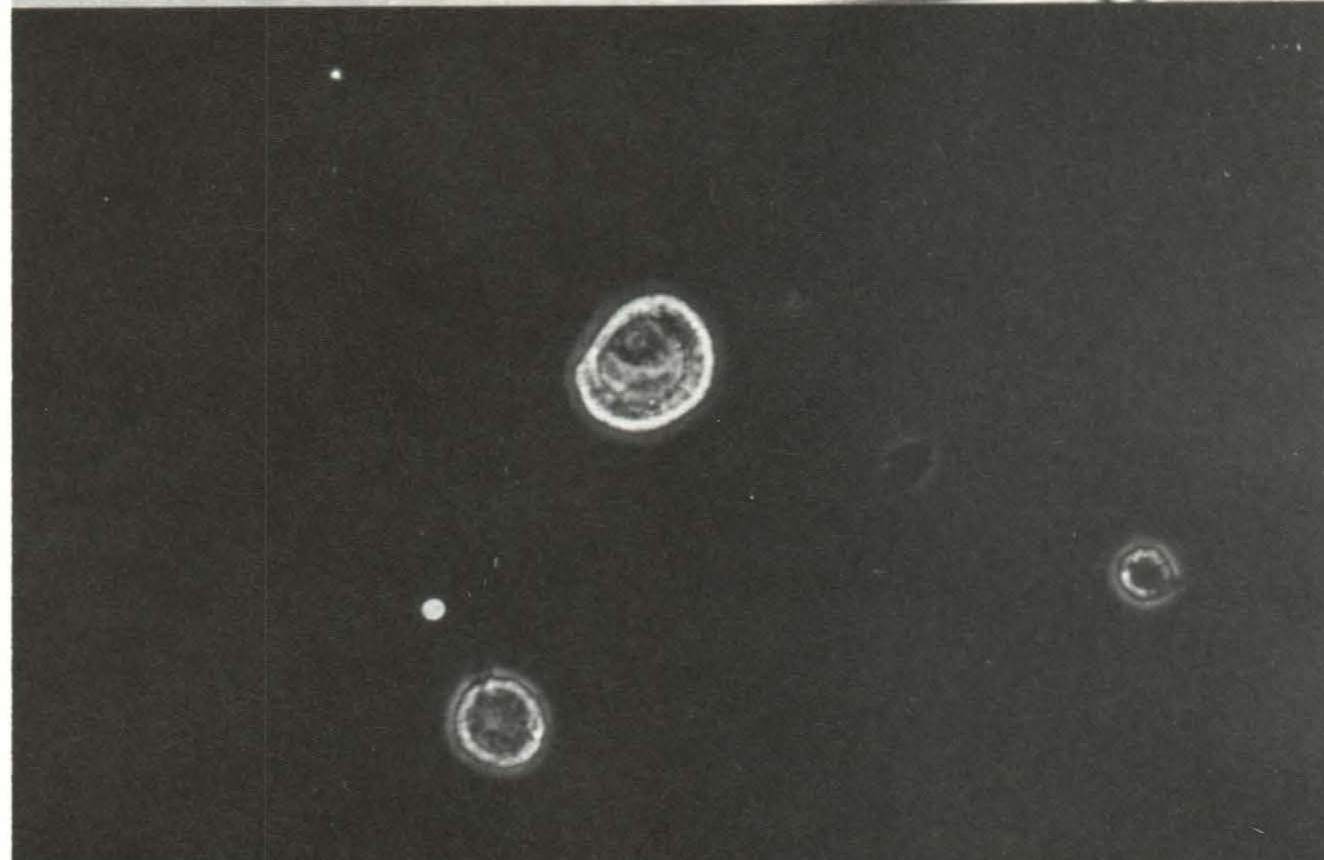
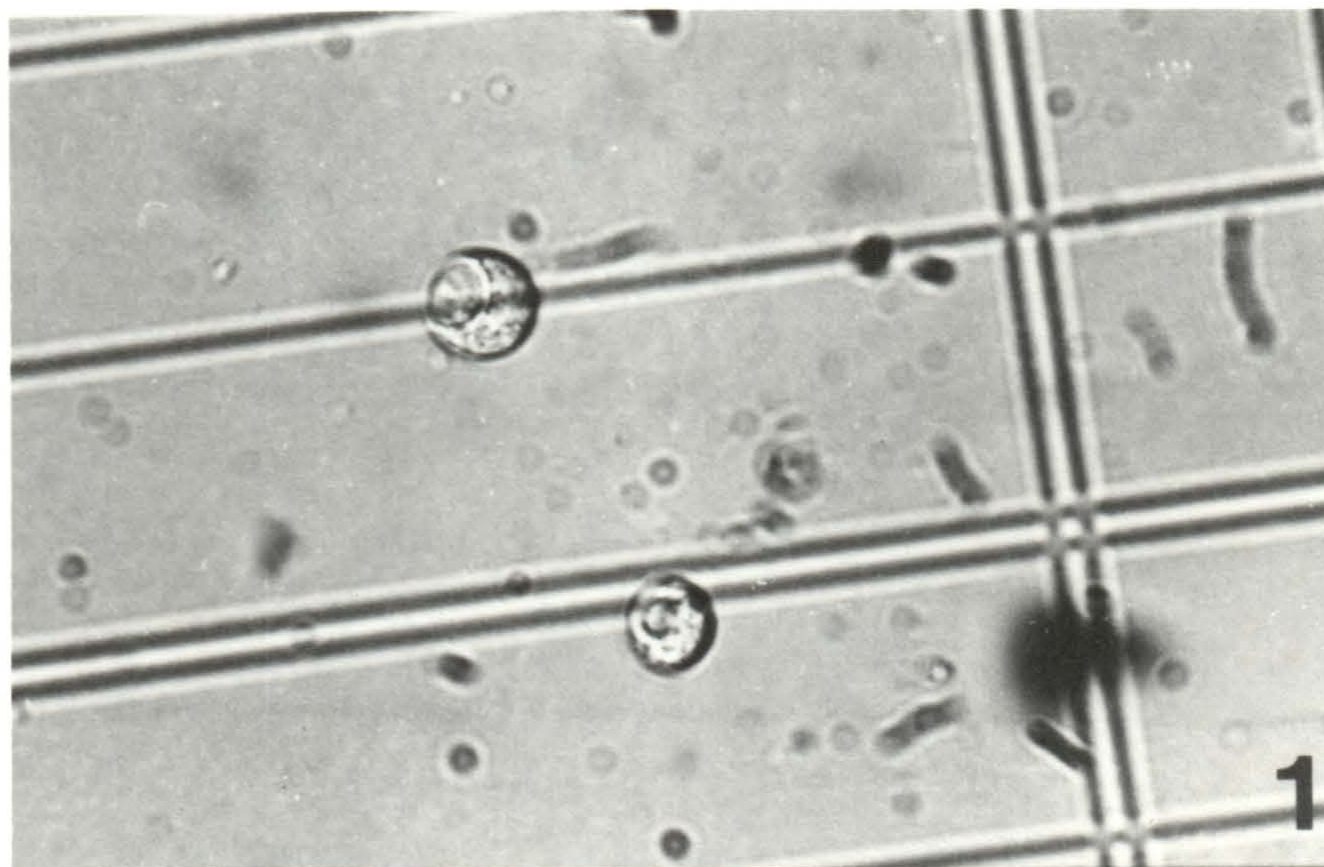




Table XXVII

Influence of viable and intact but nonviable protoplasts of *Entomophthora egressa* on hemolymph protein of spruce budworm larvae in Rms

0.018 ± 0.001 — <sup>a</sup>	0.018 ± 0.000 0.024 ± 0.003 <sup>d</sup>	— <sup>b</sup> —
0.035 ± 0.001	0.038 ± 0.001	0.041 ± 0.014
0.053 ± 0.001	— <sup>c</sup>	0.050 ± 0.013
0.066 ± 0.003	0.066 ± 0.001	0.068 ± 0.005
0.080 ± 0.001	0.078 ± 0.002	— <sup>f</sup>
0.111 ± 0.006 — <sup>b</sup>	— <sup>c</sup> 0.122 ± 0.012	— <sup>c</sup> 0.122 ± 0.001
0.154 ± 0.001 — <sup>b</sup>	— <sup>c</sup> 0.161 ± 0.000	— <sup>c</sup> 0.181 ± 0.020
0.239 ± 0.004	— <sup>c</sup>	0.246 ± 0.013
0.266 ± 0.004 — — <sup>b</sup>	— <sup>c</sup> 0.289 ± 0.001 <sup>d</sup>	0.279 ± 0.011 —
0.376 ± 0.011	0.322 ± 0.001 — <sup>c</sup>	0.316 ± 0.012 0.364 ± 0.006
0.438 ± 0.003 — <sup>b</sup>	0.435 ± 0.015 0.475 ± 0.002	0.446 ± 0.002 0.488 ± 0.005
0.505 ± 0.009		
0.526 ± 0.004	0.521 ± 0.001	0.528 ± 0.001
0.553 ± 0.005	0.540 ± 0.004	— <sup>e</sup>
0.566 ± 0.008	— <sup>c</sup>	— <sup>c</sup>
0.580 ± 0.003 —	0.572 ± 0.004 0.590 ± 0.004 <sup>d</sup>	0.585 ± 0.009 0.628 ± 0.002 0.677 ± 0.001 <sup>e</sup>

<sup>a</sup>Significantly different from adjacent values between columns

cont.

## Table XXVII cont.

<sup>b</sup>Bands not present in control gels but present in hemolymph exposed to viable and nonviable protoplasts

<sup>c</sup>Bands in control gels but absent from hemolymph exposed to viable and nonviable protoplasts

<sup>d</sup>Unique to hemolymph exposed to viable protoplasts

<sup>e</sup>Unique to hemolymph exposed to nonviable protoplasts

<sup>f</sup>Absent from hemolymph exposed to nonviable protoplasts

(Note: Only sixth instar female larvae used)



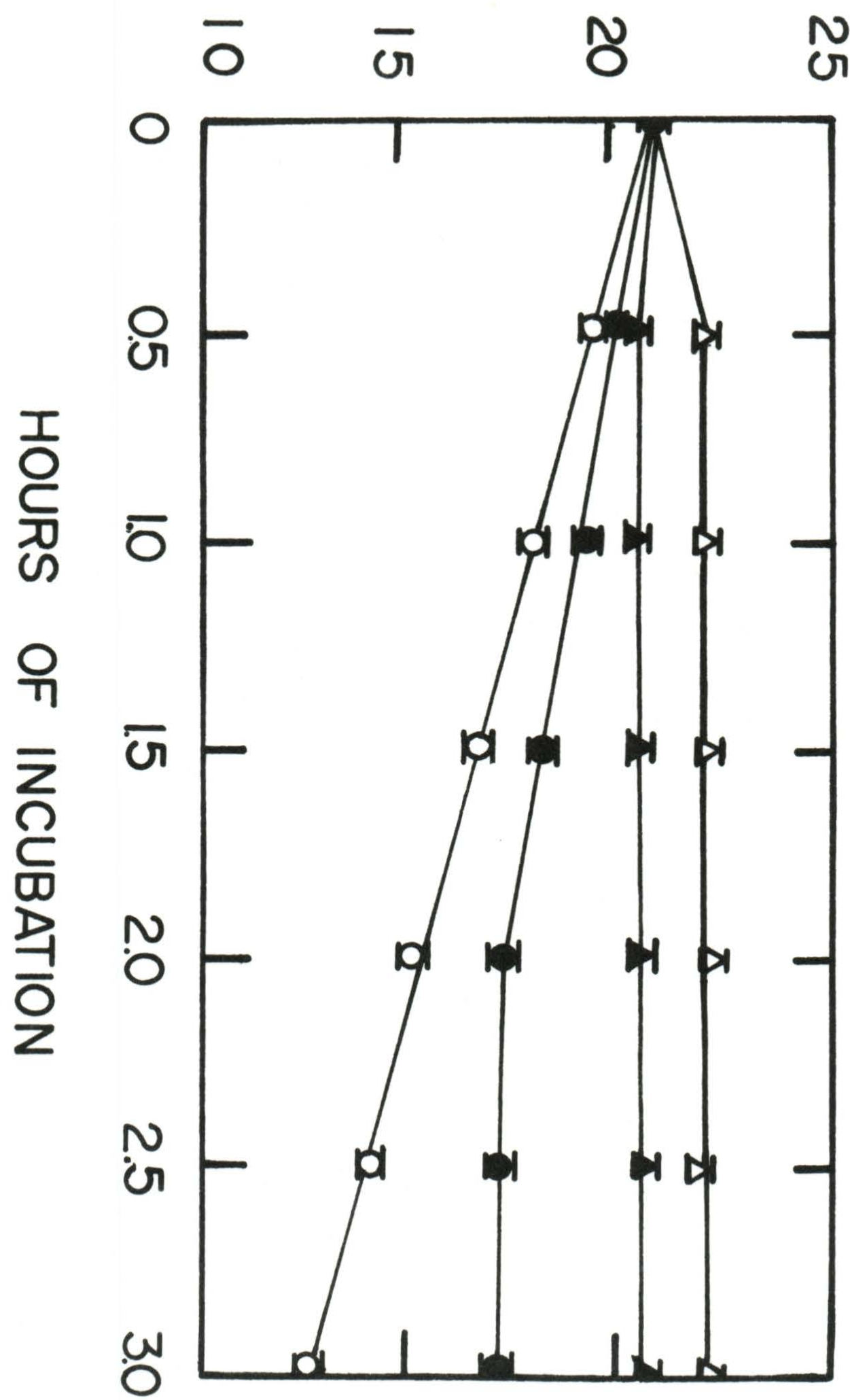
cell membrane or a protease leakage from the cell. To test this hypothesis intact viable and nonviable protoplasts, protoplast lysate and heated protoplast lysate were added to larval serum and the total protein levels determined after selected periods of incubation.

A significant reduction in total serum protein was detected at 0.5h for serum samples with intact protoplasts (viable and nonviable) and protoplast lysate (Fig. 37). The greatest reduction in serum protein level occurred with living protoplasts and protoplast lysate, in that order. The decline continued for viable protoplasts throughout the study. The reduction with protoplast lysate stopped after 2h. Nonviable protoplasts did not detectably lower the total serum protein level after 0.5h. Heated protoplast lysate raised the total protein level up to 23.5ug/ul by 0.5h, thereafter the level remained constant (Fig. 37).

Fig. 37. Effects of viable (○) and nonviable (▲) protoplasts and protoplast lysate (●) and heated protoplast lysate (Δ) on the total serum protein concentrations.



TOTAL LARVAL SERUM PROTEIN ( $\mu\text{g}/\mu\text{l}$ )



B. Discussion. The fluctuations in protein bands between larval collections may represent genetic variability and/or nutritional variability. The spruce budworm colony at Sault Ste. Marie was periodically exposed to new genetic material (Personel communication from Dale Grisdale, Forest Pest Management Institute, Sault Ste. Marie, Ontario). Because of the rearing schedule and shipping different stocks of larvae genetic heterogeneity among collections was expected. The supply of wheat germ, a component in the growth medium, was obtained from several suppliers and may have varied in nutritional value. That diet and genetic factors dramatically influence hemolymph protein electropherograms has been reported by Chen (1971).

The reduction in total hemolymph protein exposed to viable protoplasts in section VII. A. 1. suggested that:

(i) the protoplasts were actively degrading hemolymph protein and/or

(ii) the proteins of the hemolymph may be adhering to the protoplasts.

The change in whole hemolymph electropherograms and serum electropherograms exposed to viable protoplasts favoured the above proposals. Strong proteolytic activity by the



protoplasts in hemolymph was observed in section VII. A. 4. and section IV. A. 2. ii. c. Hypoproteinemia in insects parasitized by viruses, bacteria, nematodes and hymenopteran parasitoids is common (Tanada and Watanabe 1971, Weiser and Lysenko 1972, Gordon et al. 1978, Smilowilz and Smith 1977). Gardner et al. (1979) reported the nonselective decrease in all hemolymph protein bands of three species of noctuids infected by B. bassiana. From the present work with E. egressa there appeared to be some selectivity in protein degradation. Unlike the previous studies which did not include early events during infection the present study considered the effects in vitro during the first few hours.

The presence of new bands in protoplast exposed hemolymph samples compared to the serum samples (section VII. A. 2.) suggested the synthesis or release of new proteins from the hemocytes. Coles (1965) and Chippendale and Kirby (1969) reported that the hemocytes of Rhodnius prolixus Stal. and P. brassicae, respectively, synthesized hemolymph proteins. Anderson and Cook (1979) detected the release of lysozyme by the hemocytes of Spodoptera eridania L. Insects have been reported to increase the levels of selected existing proteins or to synthesize de novo antibacterial elements in response to bacterial infections (Gingrich 1964, Hinks and Briggs 1968, Chadwick and Aston 1976b, Bowman et al 1978).



The results using viable and nonviable protoplasts in hemolymph substantiated the author's initial proposals that some serum proteins adhere to the protoplast surface membrane. The viable protoplasts favored the absence of more bands than did the nonviable protoplasts which may reflect surface proteolytic enzymes on the former.

Because the protoplast lysate induced the decline in total protein until 2h and the nonviable intact protoplasts did not lower the level past 0.5h, no significant leakage of proteolytic enzymes from the latter was believed to have occurred. The fact that the protein level of the heated lysate samples increased to a constant level is consistent with the presence of hydrolytic enzymes in the nonheated protoplast lysate.



VIII. Effects of larval serum on protoplasts of *Entomophthora egressa* isolate 521.

Because I521 was initially isolated from the spruce budworm it was decided to use I521 to analyze the effects of spruce budworm larval serum on the protoplast isolate. The problem of obtaining sufficient amounts of hemolymph made a parallel study with I458 impossible.

A. Results.

1. Short-term exposure of the protoplasts to female larval serum. The protoplasts exposed to 50% larval serum for 0.5h and 1h grew, when placed in MGM, at comparable rates (3.5h/generation) and achieved equal maximum levels ( $3.5 \times 10^5 \pm 0.7 \times 10^5$  protoplasts/ml) (Fig. 38). Protoplasts from the 0.5h exposure revealed a plateau period in MGM of 3h (Fig. 38) during which no cell wall regeneration occurred. In the 1h samples cell wall regeneration occurred immediately after 39h incubation in MGM.

The growth rates of the protoplasts exposed to PTU and MGM (Fig. 38) for 0.5h (3.2h/generation) were equal to the rate for protoplasts in this medium for 1h (Fig. 39) and equal to the rate in the MGM control (Fig. 38). Except for the 1h MGM plus PTU exposed protoplasts, the proto-

Fig. 38. Effects of exposing protoplasts of Entomophthora  
egressa isolate 521 to 50% larval serum for 0.5h  
(○) and 1.0h (●), MGM without PTU (control) (△)  
and MGM plus PTU for 0.5h (◊).



PROTOPLAST CONCENTRATION (CELLS/ML  $\times 10^4$ )

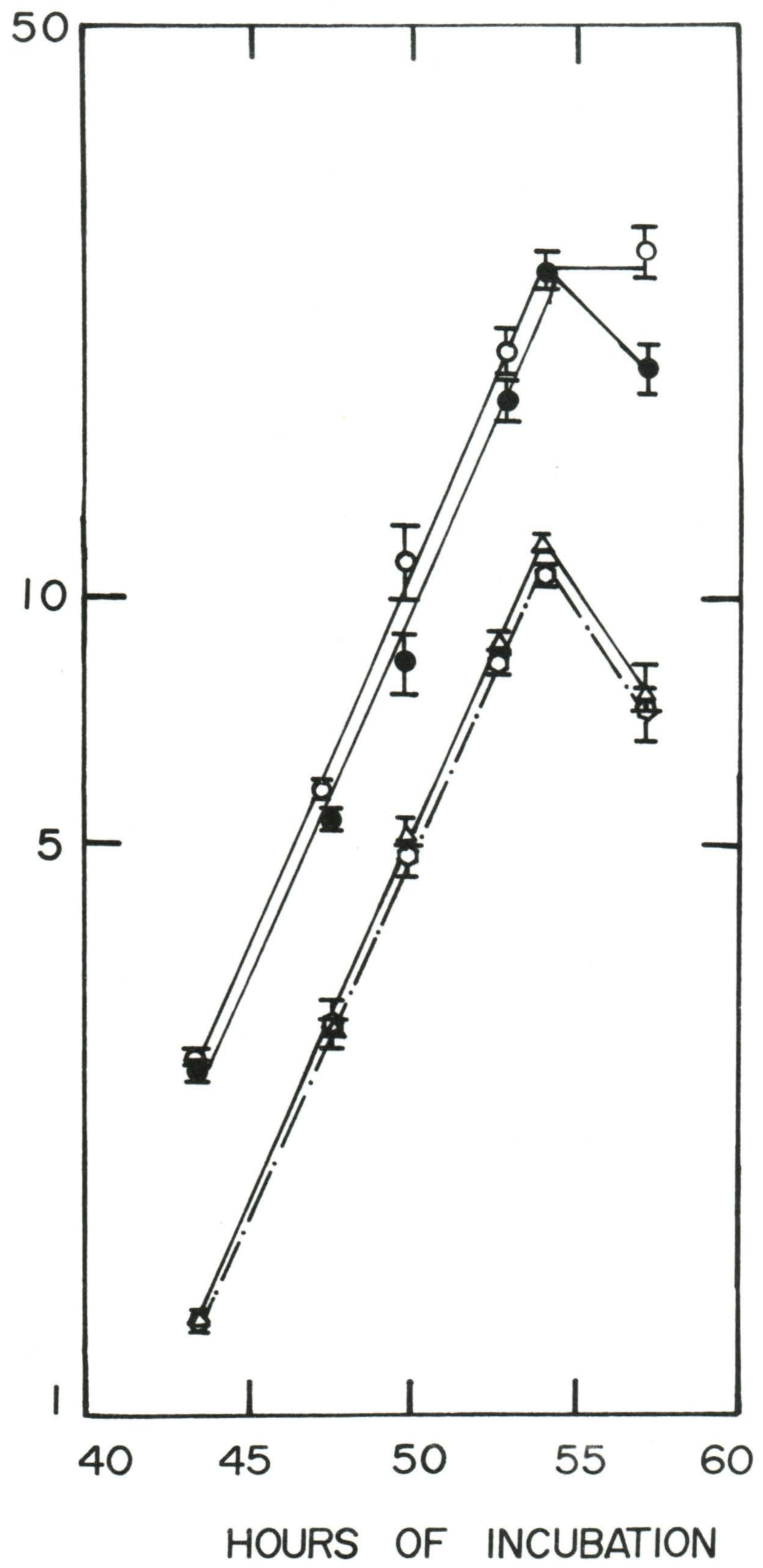
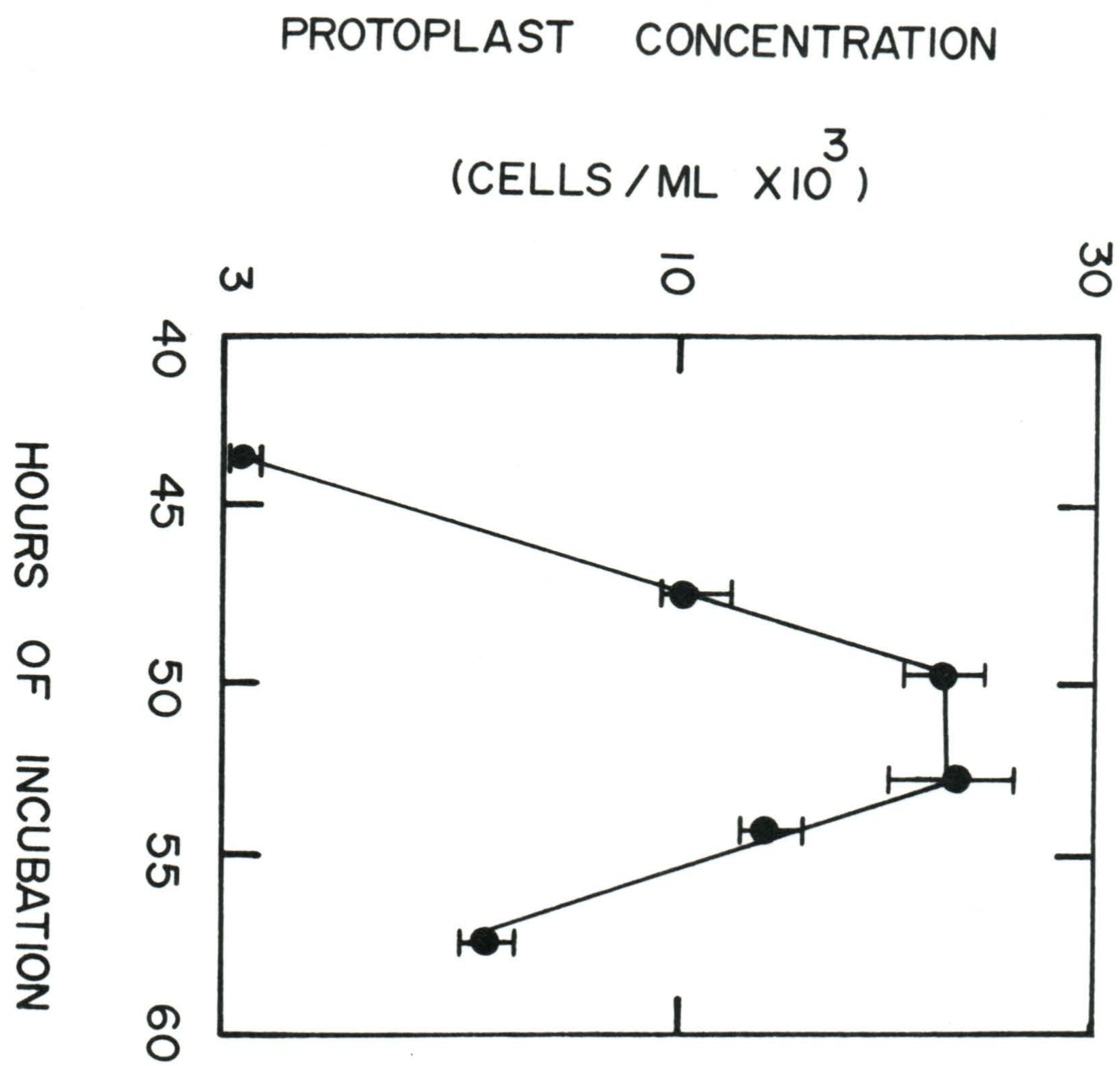


Fig. 39. Effects of exposing protoplasts of Entomophthora  
egressa isolate 521 to MGM plus PTU for 1.0h (●).





plasts in all other media for both exposure times achieved maximum levels at 53h. The former treatment favoured maximum protoplast levels at 49.5h. Serum treatment favoured greater protoplast levels throughout the growth period than did MGM plus PTU or MGM alone. Protoplasts exposed to MGM plus PTU for 0.5h produced levels comparable to the MGM control; whereas, prolonged exposure to the former medium lowered the protoplast levels.

The results using 3% serum and correspondingly diluted MGM containing PTU were not as clearly defined as the results in more concentrated media. The serum exposure for 0.5h and 1h enhanced protoplast growth rates equally (4h/generation) and to equal levels early in the growth period (Fig. 40). However, after 49.5h the protoplasts exposed to the serum for 1h declined in growth rate (8h/generation). Cells exposed to MGM with PTU for 0.5h grew at rates equal to the rate of the MGM control (3.3h/generation) and achieved comparable levels of protoplasts (Fig. 41). Cells in the former medium for 1h grew at the same rate but for a shorter period. There was an 8h plateau devoid of protoplast regeneration.

Comparison of the yields of protoplasts treated with 3% and 50% serum showed that the more dilute serum favoured more protoplasts than the more concentrated serum.



Fig. 40. Growth of the protoplasts of Entomophthora egressa isolate 521 after exposure to 3% larval serum for 0.5h (○) and 1.0h (●) and MGM (▲).

PROTOPLAST CONCENTRATION ( CELLS/ML X 10<sup>4</sup> )

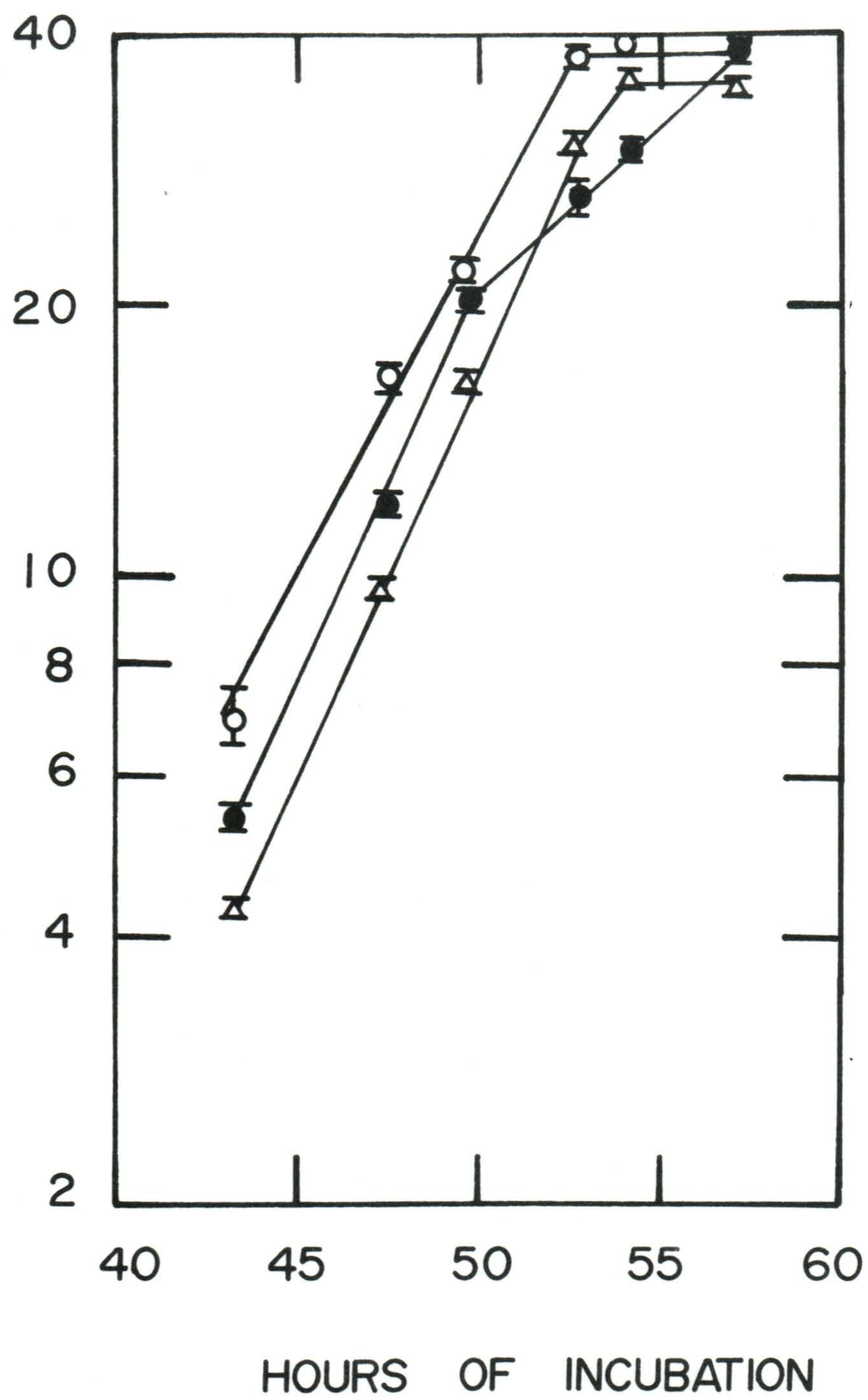
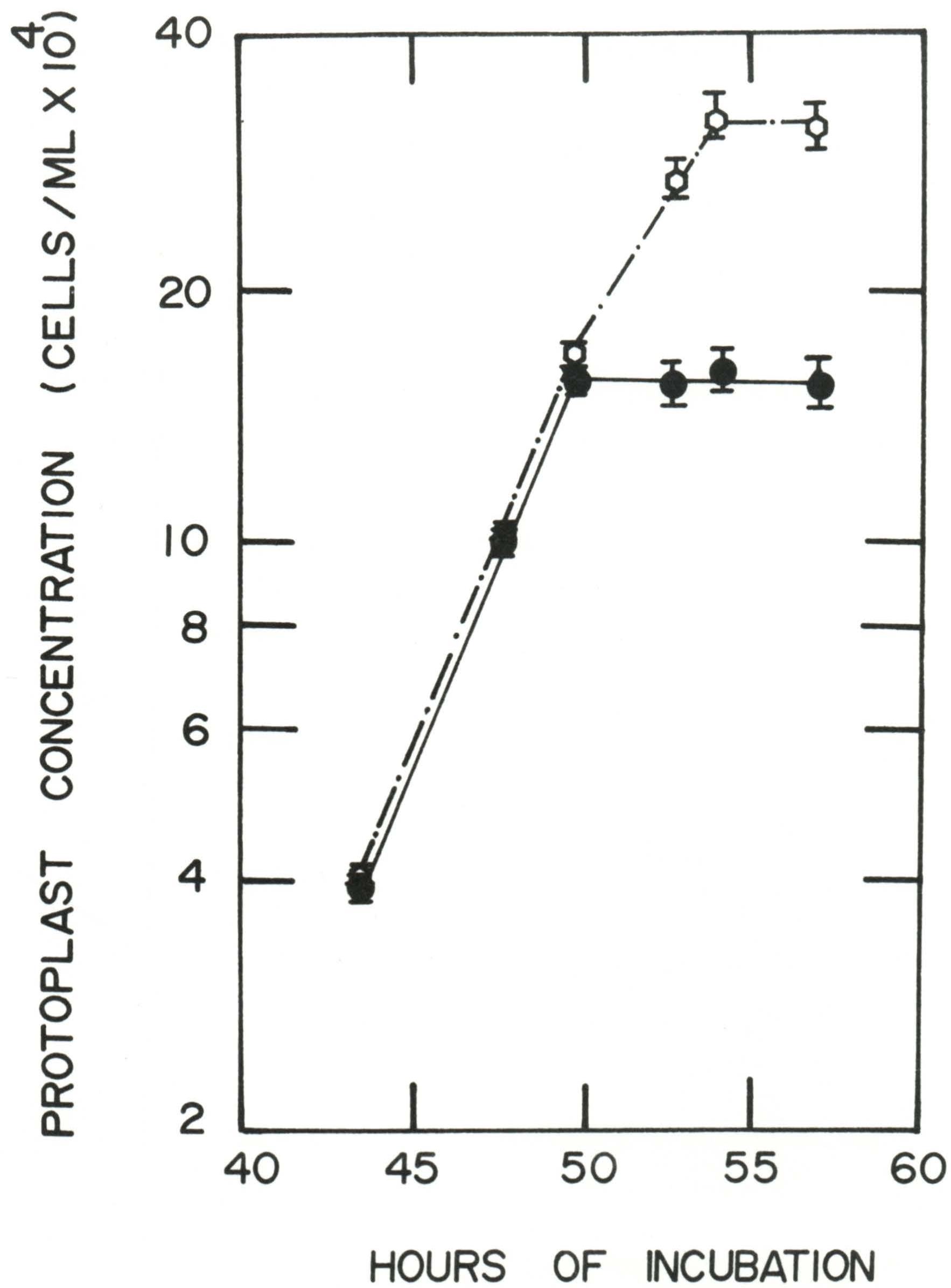




Fig. 41. Effects of PTU on the growth of protoplasts of Entomophthora egressa isolate 521 after incubation in MGM plus PTU for 0.5h (○) and 1.0h (●).





2. Long-term exposure of the protoplasts to female and male larval serum. The short-term studies indicated that larval serum favoured protoplast growth. Long-term exposure studies would be more reflective of the situation in vivo.

Exposing protoplasts to female larval serum at 1%, 2% and 3% levels for 1h revealed that the serum enhanced chain length above the level of the control samples (Fig. 42). With increasing serum concentration the stimulatory effect diminished. There was no evidence of melanization.

Prolonged (3h) exposure to female larval serum produced results similar to the 1h exposure results (Fig. 42). Comparing the 1h and 3h female larval serum results for a given serum level revealed that prolonged incubation lowered the number of protoplasts per chain. Chains were observed dissociating into individual protoplasts.

Protoplasts added to MGM containing 1%, 2% or 3% male or female larval serum failed to grow (Figs. 43 and 44). The cells remained as elliptical protoplasts for a duration related to the serum concentration. The control MGM lacking larval serum supported a 6.6 fold increase in protoplasts. All serum based media showed increasing melanization throughout the study (Fig. 45). The protoplasts in the melanized

Fig. 42. Effects of long-term exposure of larval serum on the number of protoplasts of isolate 521 of Entomophthora egressa per catenulate chain. Female larval serum for 1h (●), 3h (○) and MGM control (▲).



NUMBER OF PROTOPLAST CELLS  
PER PROTOPLAST CHAIN

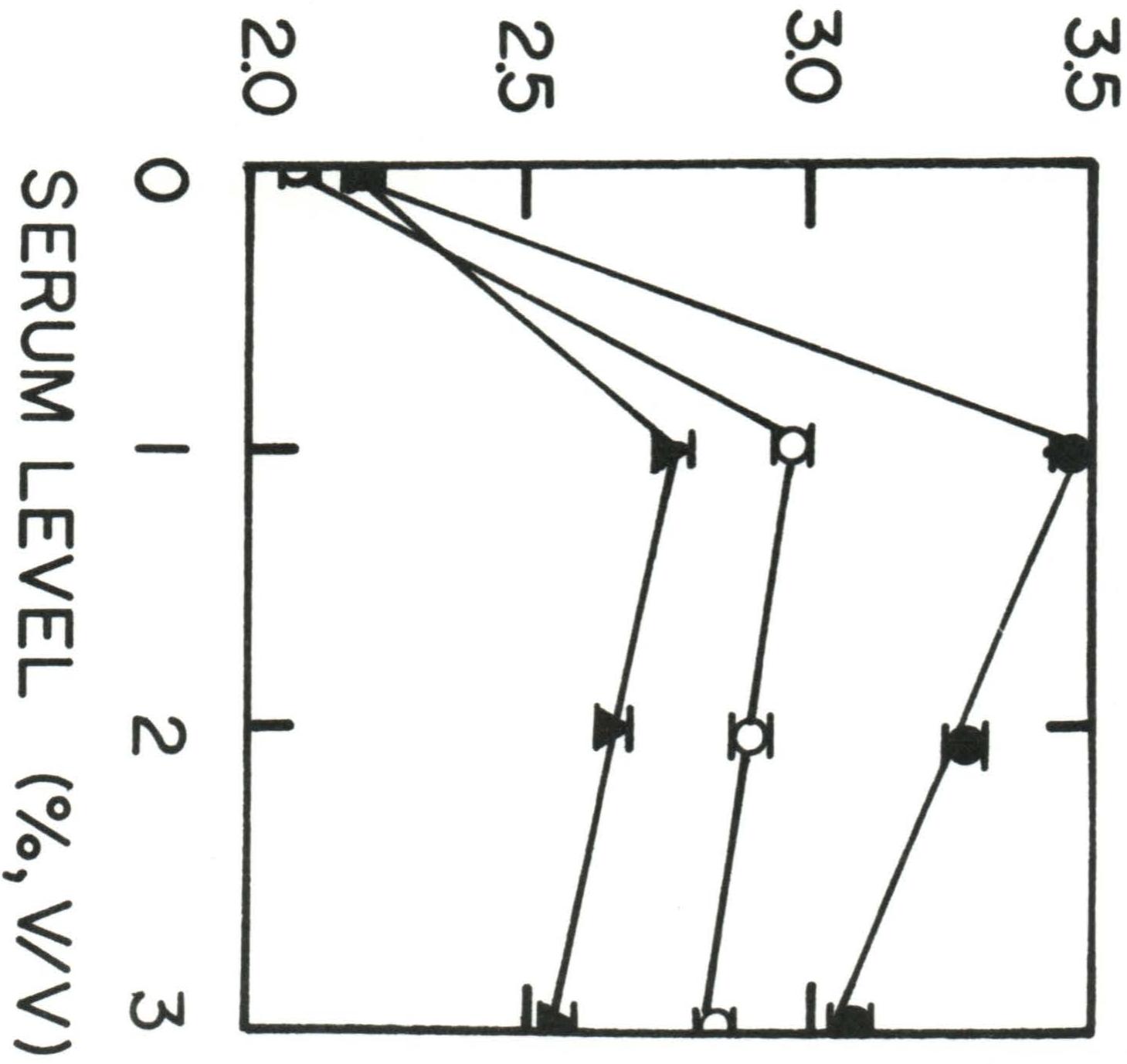


Fig. 43. Protoplast growth in MGM (O) and female larval serum at 1% (●), 2% (▲) and 3% (△) levels.

Fig. 44. Protoplast growth in MGM (O) and male larval serum at 1% (●), 2% (▲) and 3% (△) concentrations.



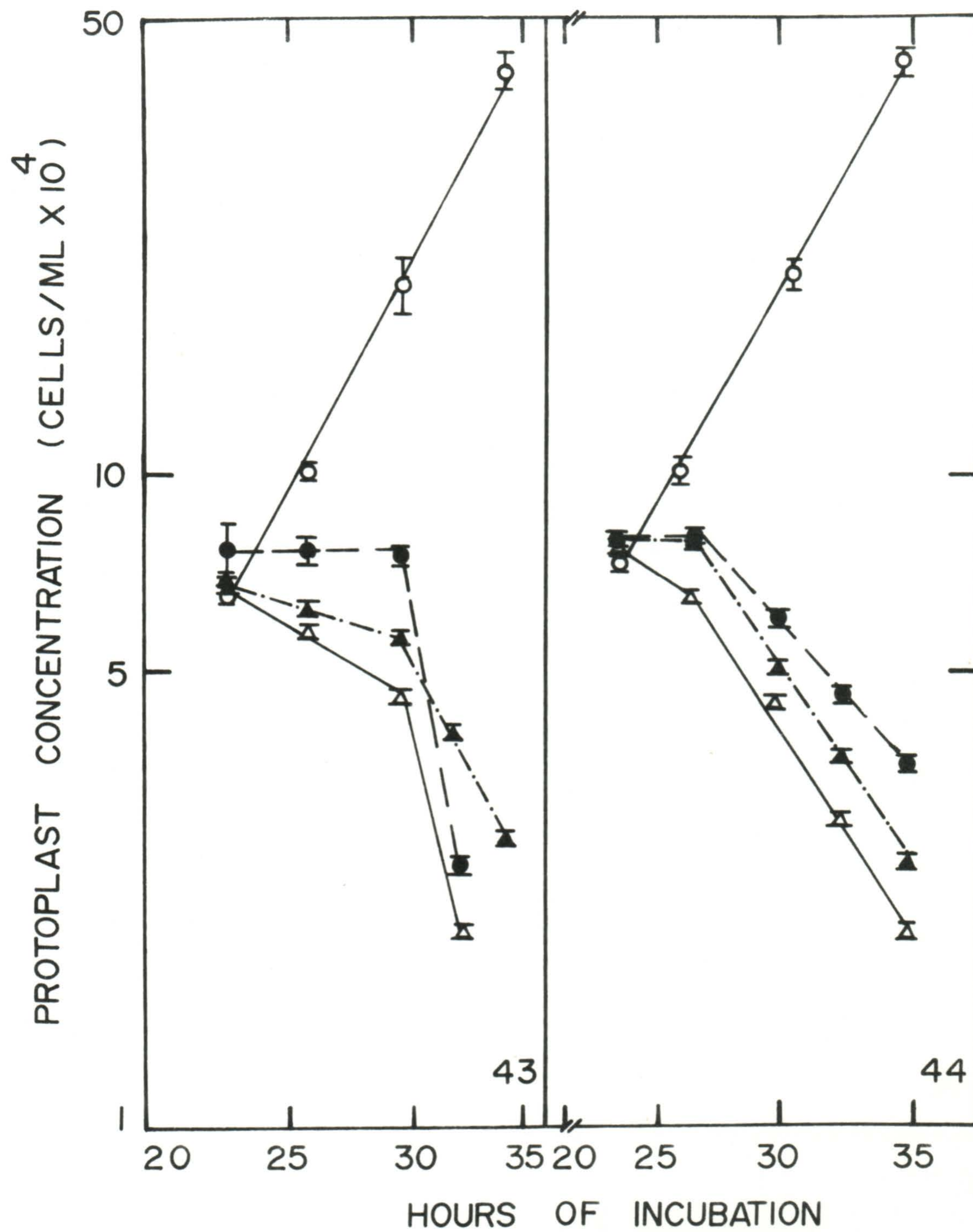
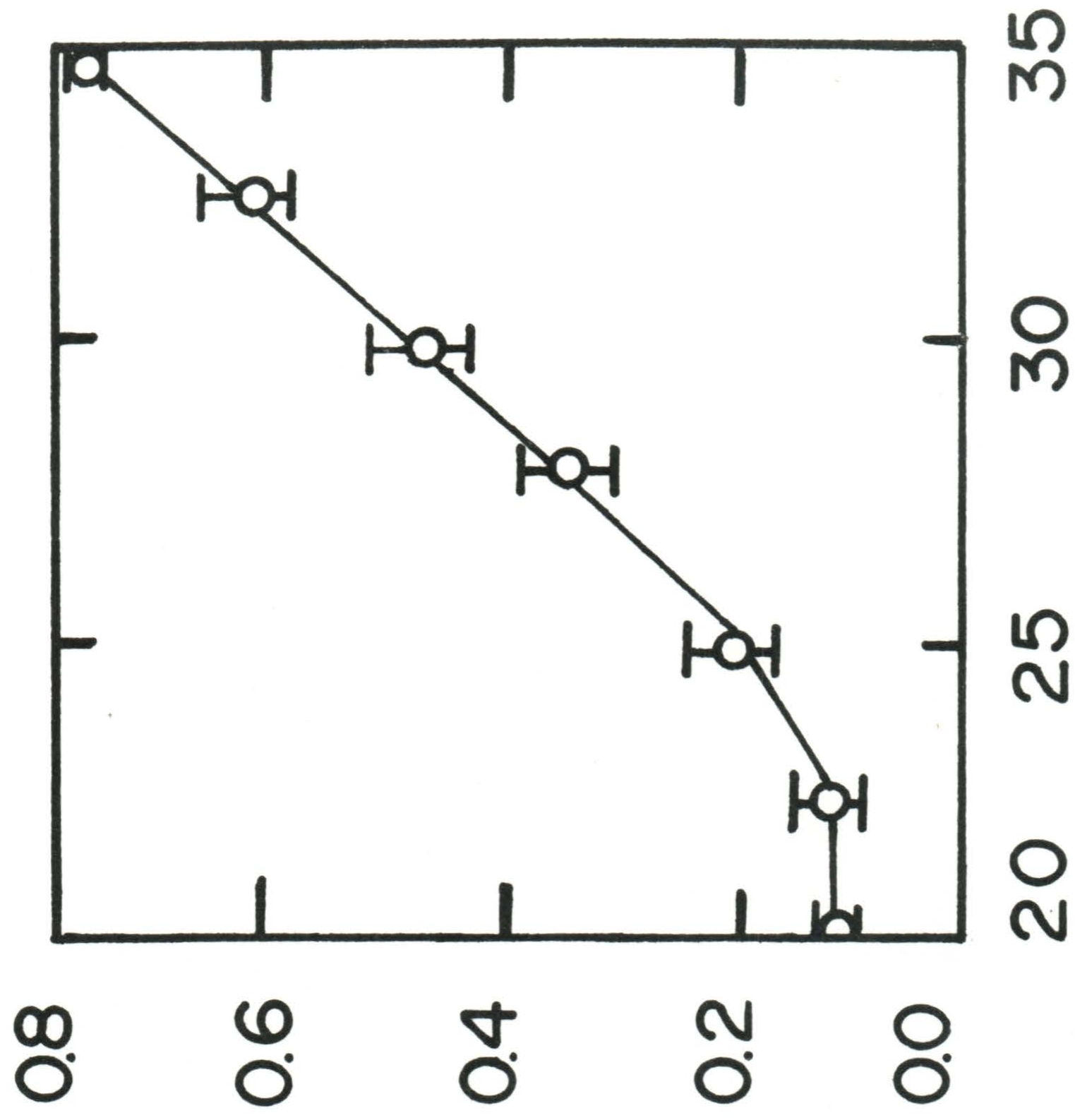


Fig. 45. Levels of melanization (pooled) in female and male based media at the selected levels of concentrations.



MELANIN ABSORPTION

(454 nm)



HOURS OF INCUBATION

media degenerated at a rate correlated with the rate of melanization (Table XXVIII).

Phenylthiourea was added to MGM and MGM with 2% larval serum to determine if melanization alone was inhibitory to E. egressa protoplasts as determined by growth rates and protoplast morphology.

The serum favoured a two step protoplast growth pattern (Fig. 46). The early growth rate in MGM plus 2% larval serum and PTU of 5h/generation was significantly less than the MGM control lacking PTU (8h/generation). Phenylthiourea in MGM inhibited protoplast growth for 21h after which cell wall regeneration occurred (Fig. 46). In both MGM plus PTU and MGM-serum plus PTU media, after achieving maximum growth at 18h and 21h, respectively, spherical hyphal bodies were detected. The larval serum based medium resulted in a second phase of protoplast activity with no detectable evidence of cell wall regeneration during this period.

Preliminary attempts to detect melanin in vivo during protoplast infection failed to reveal detectable melanin.



Table XXVIII

Correlation coefficients for the degeneration  
of Entomophthora egressa protoplasts in MGM<sup>a</sup>  
containing various levels of larval serum

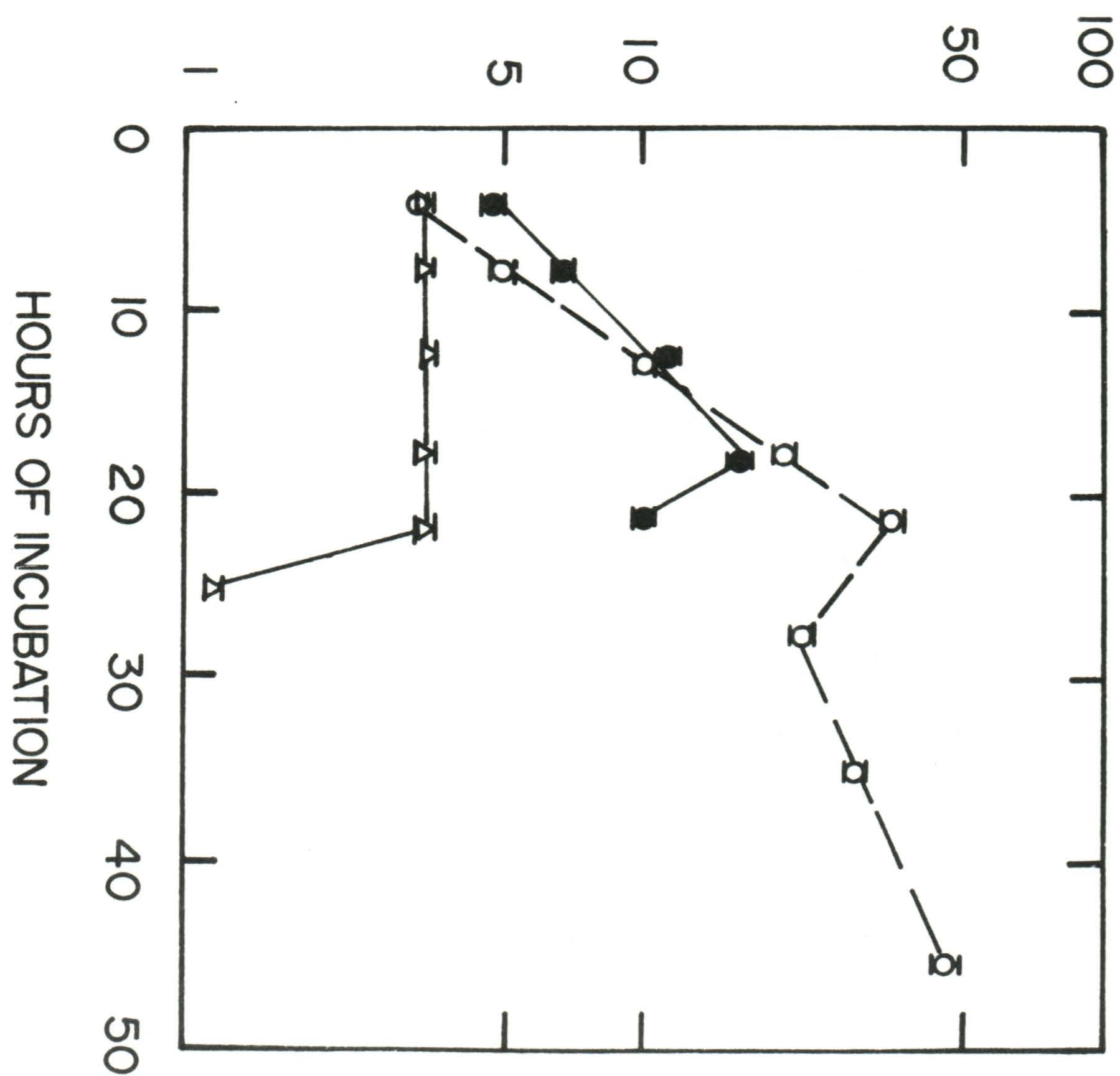
Sex	Serum level (%)		
	1	2	3
Female	0.973 (P<0.05)	0.992 (P<0.05)	0.998 (P<0.05)
Male	0.981 (P<0.05)	0.989 (P<0.05)	0.996 (P<0.05)

<sup>a</sup>Modified Grace's insect tissue culture medium

Fig. 46. Growth of protoplasts of Entomophthora egressa in 2% female larval serum with PTU (○), MGM only (●) and MGM plus PTU (Δ).



PROTOPLAST CONCENTRATION  
(CELLS / ML X 10<sup>4</sup>)



B. Discussion. Phenylthiourea was toxic to the protoplasts when added to MGM. This was not evident for the short-term exposure or long-term exposure studies when larval serum was used.

In the short-term studies the different protoplast levels between the different exposure times in larval serum and MGM may reflect differences in the early period of growth prior to the growth curve analysis. The MGM plus PTU levels after 1h exposure may reflect reduced protoplast viability and/or slower early growth rate compared to the MGM control levels.

That the exposure to 50% larval serum did not induce as high a protoplast level as the 3% larval serum, in view of the absence of any protoplast toxicity by PTU in the serum, this would argue for antiprotoplast activity in the serum. This was further supported by the effects of increasing serum levels on the number of protoplasts per chain in the long-term studies. Possibly some of the proteins (section VII. 1.) reported to adhere to the protoplast cell membrane may be involved. That the chain length of protoplasts in larval serum were longer than protoplasts in MGM suggests that the serum contained elements either utilized by the protoplasts and/or inhibited protoplast dissociation. Tyrrell (1977) reported that long protoplast "chains" occurred naturally in



spruce budworm larvae.

Failure of the larval serum-MGM medium lacking PTU to support protoplast growth and the correlation with melanin implicates melanin or its precursors as toxic to the protoplasts. This was further substantiated by the growth in serum medium when PTU was added.

The preliminary in vivo studies, if valid, would lead one to conclude that melanin production did not play a role in the inhibition of E. egressa protoplasts.

Unestam and co-workers (1970, 1972, 1975, 1976) reported that the growth of A. astaci in vivo in several resistant species of crayfish was slowed by melanization of the fungal wall. Effective inhibition of the growth of B. bassiana by melanization has been documented for humoral encapsulation in species of Chironomus larvae (see Nappi 1975).



IX. Growth of *Entomophthora egressa* protoplasts in media based on male and female larval serum NPC.

Because of the problem of obtaining sufficient amounts of larval serum for growth studies an attempt was made to simulate the larval serum. In the following experiments the NPC reported in the larval serum were used to prepare an artificial medium to simulate the larval serum.

A. Results.

1. NPC levels of the male and female sixth instar spruce budworm larvae. The major NPC (5mM) in both sexes were phosphoethanolamine, L-proline, L-lysine, L-histidine, glycine and L-glutamine (Tables XXIX and XXX). Moderate levels (1mM-4.9mM) of NPC included taurine, L-threonine, L-serine, L-asparagine, L-glutamine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine and  $\beta$ -alanine. There was no significant difference between the level at a given NPC in either sex ( $P > 0.05$ ) nor between the total NPC of either sex ( $t = 0.550$ ,  $P > 0.6$ ). An unidentified peak eluted in the sarcosine region for all samples of both sexes. Acid hydrolysis of the samples removed this peak.

Although there were no statistical differences in larval



Table XXIX

Level of NPC<sup>a</sup> in the serum of male  
sixth instar spruce budworm larvae

NPC	Concentration	
	mM <sup>b</sup>	mg/L <sup>c</sup>
Cysteic acid/phosphoserine	0.109 $\pm$ 0.017	9.0/10.0 <sup>d</sup>
Glycerophosphoethanolamine	trace	0.2
Phosphoethanolamine	7.502 $\pm$ 1.589	1058.0
Taurine	1.664 $\pm$ 0.126	208.0
Methionine sulfoxide	0.626 $\pm$ 0.108	103.0
L-Hydroxyproline	0.944 $\pm$ 0.120	124.0
L-Aspartic acid	0.095 $\pm$ 0.010	13.0
L-Threonine	3.302 $\pm$ 0.154	393.0
L-Serine	3.286 $\pm$ 0.144	2109.0
L-Glutamine	14.430 $\pm$ 0.983	
L-Asparagine	2.199 $\pm$ 0.680	291.0
L-Proline	23.911 $\pm$ 1.967	2762.0
L-Glutamic acid	1.130 $\pm$ 0.104	166.0
L-Citrulline	0.267 $\pm$ 0.149	47.0
Glycine	9.279 $\pm$ 0.708	696.0
DL-Alanine	3.224 $\pm$ 0.633	287.0
$\alpha$ -Amino-n-butyric acid	0.053 $\pm$ 0.000	5.0
L-Arginine	2.181 $\pm$ 0.119	380.0
Halfcystine	0.005 $\pm$ 0.000	— <sup>f</sup>
L-Valine	2.953 $\pm$ 0.206	346.0
L-Cystathionine	0.186 $\pm$ 0.093	41.0
L-Methionine	1.376 $\pm$ 0.052	205.0
L-Isoleucine	1.047 $\pm$ 0.030	137.0

cont.

Table XXIX cont.

NPC	Concentration	
	mM	mg/L
L-Leucine	1.581 $\pm$ 0.101	207.0
L-Tyrosine	3.924 $\pm$ 0.440	711.0
L-Phenylalanine	1.262 $\pm$ 0.107	208.0
B-Alanine	0.867 $\pm$ 0.196	78.0
L-Hydroxylysine	0.104 $\pm$ 0.046	21.0
$\gamma$ -Aminobutyric acid	0.042 $\pm$ 0.009	4.0
L-Ornithine	0.476 $\pm$ 0.074	63.0
L-Ethanolamine	0.452 $\pm$ 0.196	28.0
Ammonia	2.233 $\pm$ 0.357	
L-Lysine	8.922 $\pm$ 1.340	1303.0
L-1-Methylhistidine	0.081 $\pm$ 0.013	14.0
L-Histidine	16.114 $\pm$ 1.166	2501.0
L-3-Methylhistidine	0.014 $\pm$ 0.000	2.0
L-Tryptophan	0.132 $\pm$ 0.034	27.0
L-Carnosine	0.412 $\pm$ 0.000	93.0
Total <sup>e</sup>	116.842 $\pm$ 10.195	—

<sup>a</sup>Ninhydrin positive compounds<sup>b</sup>Level in the larval serum based on 6 samples<sup>c</sup>Level in the medium based on larval serum<sup>d</sup>9.0mg/L cysteic acid and 10.0mg/L phosphoserine<sup>e</sup>Minimum total NPC excluding 1 unknown and ammonia<sup>f</sup>Not used



Table XXX  
 Level of NPC<sup>a</sup> in the serum of female  
 sixth instar spruce budworm larvae

NPC	Concentration	
	mM <sup>b</sup>	mg/L <sup>c</sup>
Cysteic acid/phosphoserine	0.112 ± 0.020	9.0/10.0 <sup>d</sup>
Glycerophosphoethanolamine	trace	0.2
Phosphoethanolamine	8.748 ± 0.185	1233.0
Taurine	1.439 ± 0.152	180.0
Methionine sulfoxide	0.580 ± 0.112	96.0
L-Hydroxyproline	0.721 ± 0.118	94.0
L-Aspartic acid	0.156 ± 0.020	21.0
L-Threonine	3.386 ± 0.124	403.0
L-Serine	3.550 ± 0.321	373.0
L-Glutamine	15.260 ± 1.482	2230.0
L-Asparagine	1.797 ± 0.487	237.0
L-Proline	21.380 ± 0.805	2453.0
L-Glutamic acid	1.699 ± 0.434	250.0
L-Citrulline	0.164 ± 0.021	29.0
Glycine	8.983 ± 0.589	674.0
DL-Alanine	2.262 ± 0.167	202.0
α-Amino-n-butyric acid	0.117 ± 0.006	12.0
L-Arginine	2.400 ± 0.124	418.0
Halfcystine	0.007 ± 0.000	— <sup>f</sup>
L-Valine	3.042 ± 0.122	356.0
L-Cystathionine	0.601 ± 0.146	133.0
L-Methionine	1.256 ± 0.189	187.0
L-Isoleucine	0.983 ± 0.147	129.0
L-Leucine	1.904 ± 0.097	249.0

cont.

Table XXX cont.

NPC	Concentration	
	mM.	mg/L
L-Tyrosine	3.710 $\pm$ 0.211	672.0
L-Phenylalanine	1.545 $\pm$ 0.061	255.0
 D-Alanine	 1.012 $\pm$ 0.270	 90.0
L-Hydroxylysine	0.140 $\pm$ 0.041	28.0
$\gamma$ -Aminobutyric acid	0.049 $\pm$ 0.022	5.0
L-Ornithine	0.376 $\pm$ 0.065	50.0
 L-Ethanolamine	 0.505 $\pm$ 0.136	 31.0
Ammonia	3.202 $\pm$ 0.450	—
L-Lysine	7.674 $\pm$ 0.773	1120.0
L-l-Methylhistidine	0.056 $\pm$ 0.014	9.0
L-Histidine	14.230 $\pm$ 0.368	2208.0
 L-3-Methylhistidine	 0.008 $\pm$ 0.001	 1.0
L-Tryptophan	0.113 $\pm$ 0.019	23.0
L-Carnosine	0.230 $\pm$ 0.069	52.0
Total <sup>e</sup>	111.000 $\pm$ 2.999	—

a, b, c, d, e, f - Refer to Table XXVIII foot notes



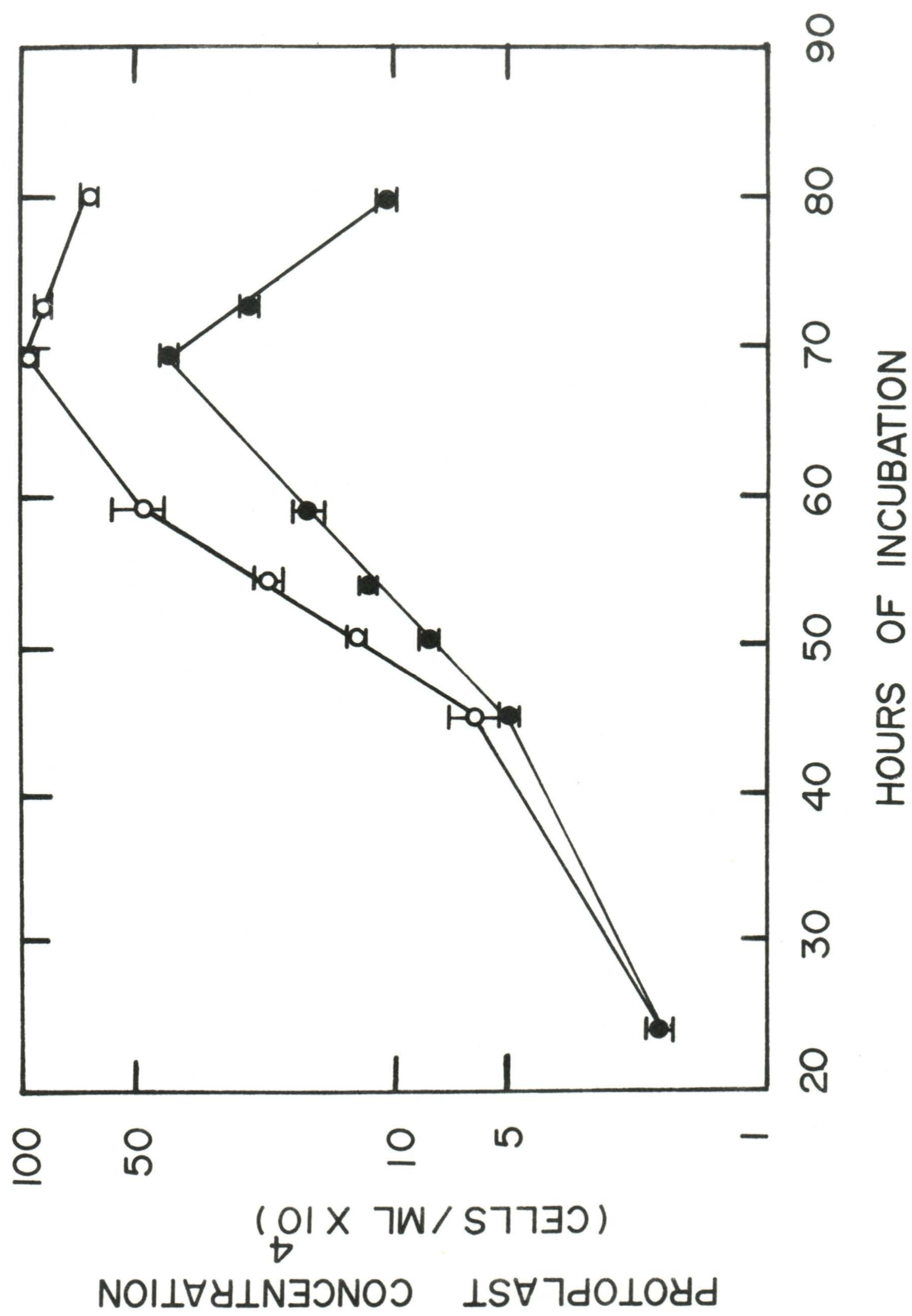
NPC for the sexes the possibility of the NPC levels having physiological significance for E. egressa protoplasts remained to be determined.

2. Protoplasts of *Entomophthora egressa* in serum simulated media. The medium based on female serum NPC (FM) and containing FCS did not support protoplast growth. The protoplasts maintained the spindle shape in the medium for 54.4h after which the cells became spherical and lysed. The "male" based medium (MM) supported protoplast growth (Fig. 47). Initially the growth rate (16h/generation) was slow but increased substantially from 45h to 8h/generation. Maximum growth occurred by 69.5h followed by spherical hyphal body formation. Diluting FM and MM by 30% with a sucrose-MES buffer did not alter the osmolality of the media but did dilute the nutrients. This was an attempt to detect possible nutrient toxicity. The diluted FM did not support protoplast growth. The diluted MM supported protoplast growth and later normal morphogenesis (Fig. 47). The population doubling time was 4.5h/generation.

3. Comparison of lobster serum and fetal calf serum dialysates and their effects on protoplast growth in selected media. Because lobsters are believed to be more closely allied to insects than fetal calves the serum of the former may approximate larval serum more than the latter.

Fig. 47. Growth of protoplasts of isolate 521 of Entomophthora egressa in medium based on male larval NPC (●) and 30% dilution (○)..





Both lobster and fetal calf serum were dialysed to reduce the chances of nonprotein components interacting with the simulated media.

The electropherograms differed dramatically between the serum sources (Plate 19, Fig. 1; Fig. 48). A comparison of  $R_m$  values revealed the absence of similar protein bands. The major bands in the lobster serum dialysate were in both the fast and slow migrating regions of the gels (Fig. 48) whereas, in the fetal calf serum dialysates, with the exception of albumin ( $R_m$  0.923), the major bands were in the middle of the gel, an area devoid of bands in the lobster samples (Fig. 48).

Both FM and MM containing the fetal calf serum dialysate favoured protoplast growth (Figs. 49 and 50). In FM the protoplasts grew to greater levels and for longer periods of time than did cells in MM. The growth profile of cells in the latter media differed in terms of growth rate, level and duration from the profile in MM with whole fetal calf serum.

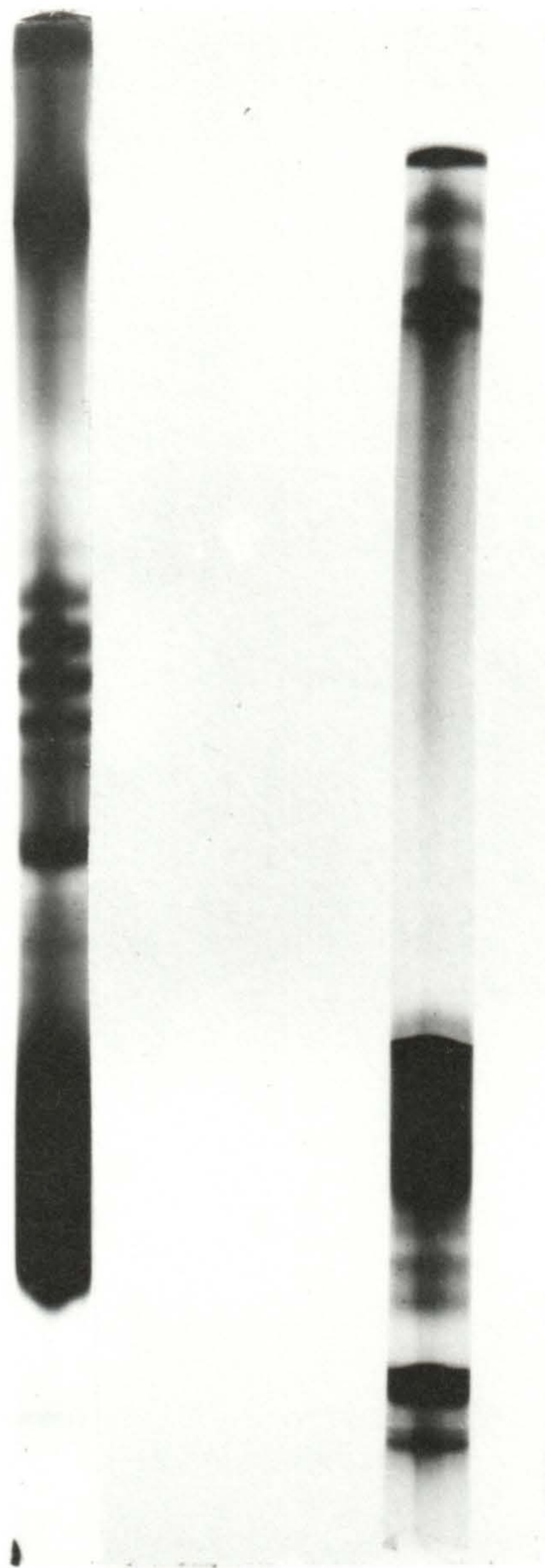
Dilution of FM by 30% resulted in a generation time significantly shorter than the nondiluted medium at 2h/generation and 6.8h/generation, respectively.

The lobster dialysate prevented protoplast growth in FM



## Plate 19

Fig. 1 Electropherogram of the dialysates of fetal calf serum (F) and lobster serum (L).



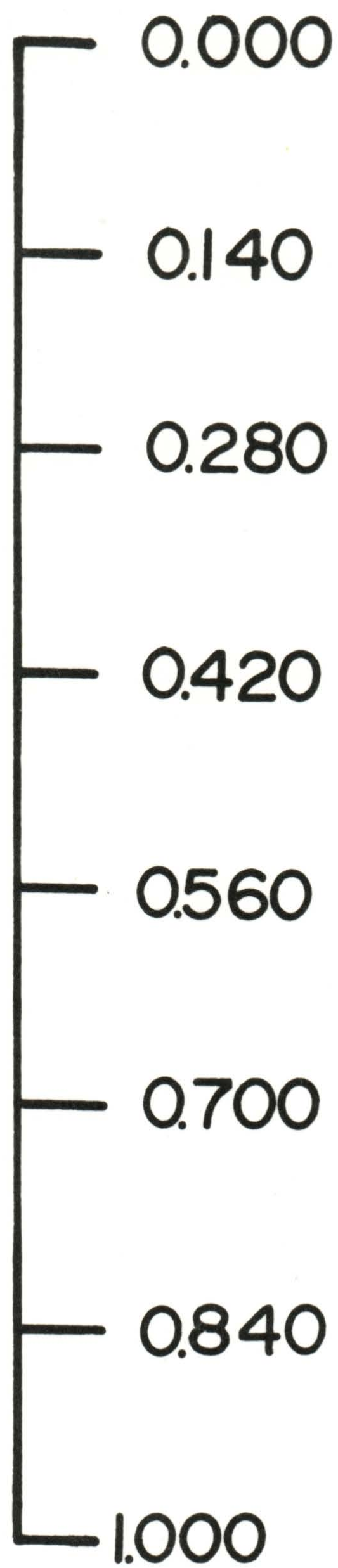
**F**

**L**



Fig. 48. Tracing of Plate 19, Fig. 1. Refer to Fig. 1  
for code.

# RM SCALE



F



L



Fig. 49. Growth of Entomophthora egressa protoplasts in male NPC based medium containing fetal calf serum dialysate (O) and lobster serum dialysate (Δ).

PROTOPLAST CONCENTRATION

(CELLS / ML  $\times 10^4$ )

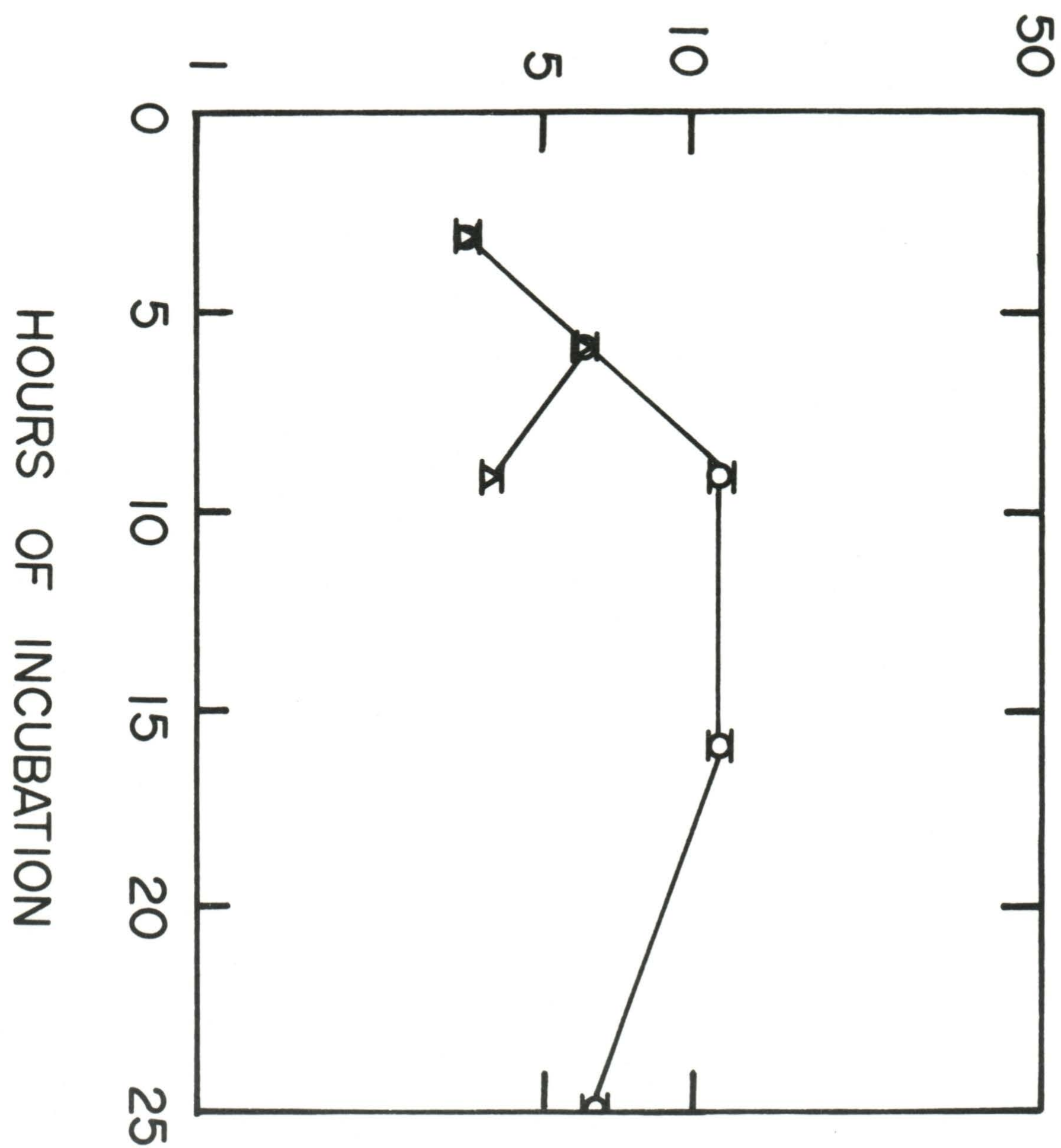
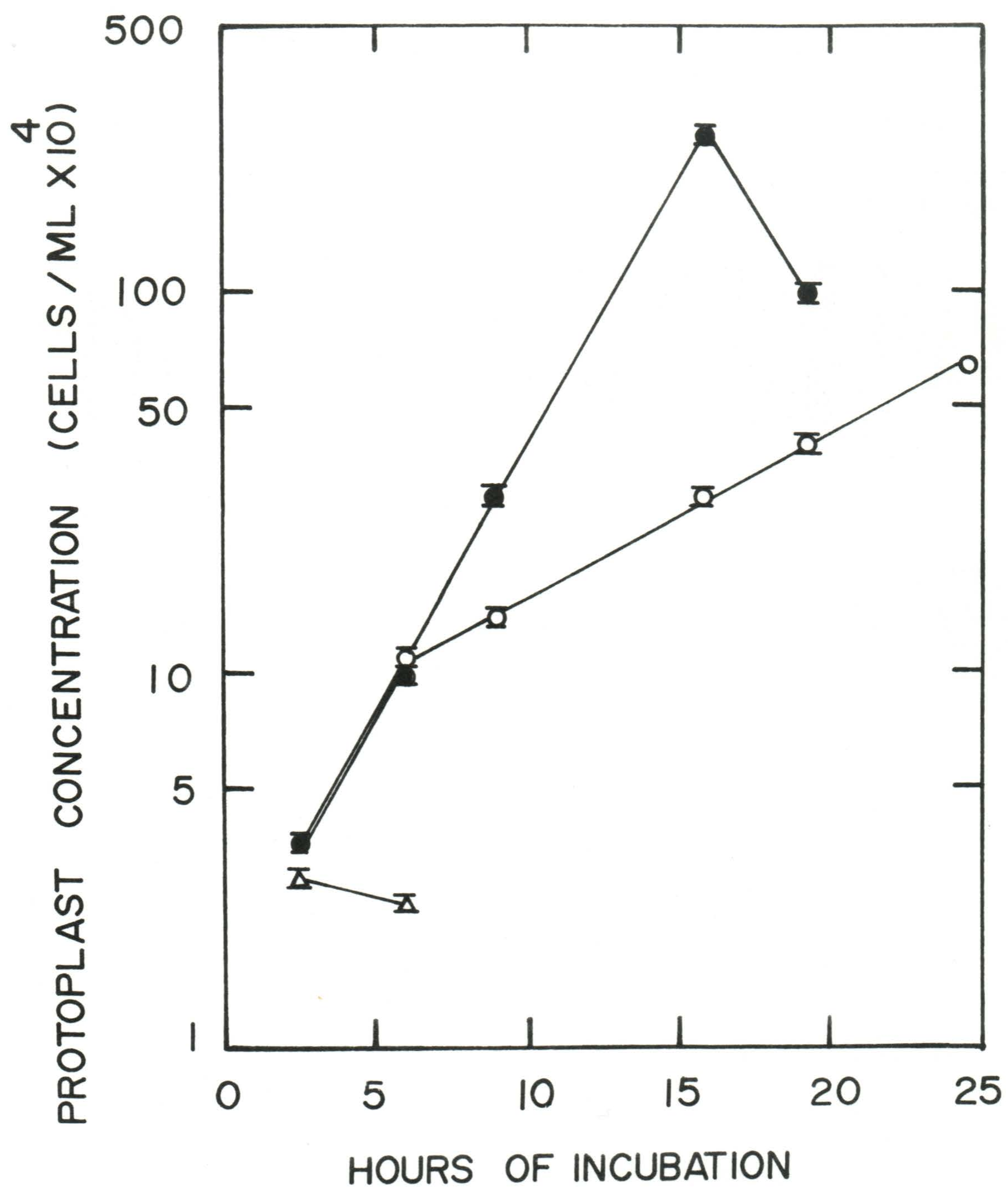




Fig. 50. Growth of Entomophthora egressa protoplasts in female NPC based medium containing fetal calf serum dialysate non-diluted (○) and diluted by 30% (●) and lobster serum dialysate (Δ).





(Fig. 50) and allowed a brief period of growth in MM (Fig. 49). In both media protoplasts became spherical and lysed within 1.5h.

Attempts to remove any putative lobster serum component binding to the protoplast cells failed. Electrophoresis of absorbed lobster serum dialysate did not reveal any detectable change in protein band width or Rm positions.

B. Discussion. It has generally been reported that insect hemolymph has high concentrations of glutamic acid and proline and lesser amounts of the basic amino acids (Boctor and Salem 1973). In the spruce budworm serum glutamine, histidine, lysine and glycine were the major NPC. Wyatt et al. (1956) reported that glutamine, histidine and lysine were the predominant NPC of B. mori larval hemolymph. High levels of histidine have been detected in the hemolymph of Spodoptera littoralis Boisduval (Boctor and Salem 1973) and L. fiscellaria fiscellaria (Dunphy et al. 1977).

The high levels of the above mentioned NPC of the spruce budworm may have a role in osmoregulation. Benassi et al. (1961) have speculated on the involvement of glycine in osmoregulation by Schistocera gregaria Forsk.

The pH of the sixth instar spruce budworm hemolymph is 6.9 (Hemipel 1956). It is conceivable that because the pKa of the imadizole ring of histidine is 6.1 the amino acid may serve as a hemolymph buffer. The pKa values of the  $\alpha$ -carboxyl and  $\alpha$ -amino groups are too far removed from hemolymph pH to provide significant buffering.

The unknown eluting in the sarcosine area for both sexes, based on the hydrolysis results, appears to be a peptide. Peptides in insect hemolymph are common, serving



as metabolites and osmoregulators (Levenbook 1966, Collette 1976a,b, Bodnaryk 1978).

There was no difference in the individual NPC levels or total NPC levels between the male and female spruce budworm larvae. There is a scarcity of data on differences in NPC levels for insect larvae. Inokuchi (1972) detected differences in the level of cystathionine between male and female B. mori larvae.

The results of protoplast growth in the different media only indirectly suggested possible differences in the suitability of male and female larval hemolymph to support protoplast growth.

The female serum-based medium with whole fetal calf serum failed to support protoplast growth; whereas, the male based medium was conducive to growth. It was apparent that fetal calf serum interacted with both FM and MM influencing protoplast growth because fetal calf serum retained in the dialysis tubing was more conducive to growth in FM than in MM. The fact that both diluted male and female serum based media favoured accelerated protoplast growth was evidence for possible partial nutritional inhibition of protoplast growth.

The inability of lobster serum components retained in the dialysis tubing to support growth in either FM or MM and induced rapid protoplast lysis suggested the following:

(i) that protein(s) reminiscent of lectins bound to the surface inducing lysis, or

(ii) that reversibly bound enzymes were responsible for lysis.

The absence of detectable changes in the electropherograms of absorbed lobster serum dialysate supports the latter contention.



X. Mycotoxins of isolates of *Entomophthora egressa*.

A. Results.

1. Description of mycotoxin induced paralysis.

The injection of 5ul of spent protoplast culture medium containing protoplasts into spruce budworm larvae induced rapid total paralysis within fractions of a second. The paralysis spread posteriorly as exhibited by the cessation of movement by the abdominal prolegs. The thoracic legs ceased movement immediately upon injection. Within 5min postinjection, the mouth parts were barely moving and only in a few larvae (1%). The larvae, in essence, froze in the position occupied during injection and remained in this position for over 100h. The body was soft and did not exhibit signs of bloating. Frass excretion ceased. The control larvae continued normal activity after injection with 6ul of MGM.

2. Toxicity of spent medium and protoplasts.

Larvae injected with centrifuge-washed protoplasts did not exhibit signs of paralysis or develop entomophthoraceous mycoses during a 242h incubation period. The larvae eventually pupated.

Both diluted and nondiluted spent MGM induced paralysis



in the larvae within 2h of injection (Table XXXI). With prolonged incubation the level of total paralysis declined and the level of partial paralysis increased until 76h after injection by which time the effects leveled off. Partial paralysis referred to the ability of larvae to move mouth parts and occasionally one or two of the prothroacic legs. The control larvae were mobile throughout the study.

The hemocoel of paralysed larvae became contaminated with Gram negative, motile, rod-shaped bacteria (Table XXXI). The rate of contamination was related to the concentration of injected spent medium in that 6 $\mu$ l injections induced septicemia sooner than 3 $\mu$ l injections of dilute medium. The percentages of larvae with bacterial infection leveled off 76h post-injection and were equal for all treatments. The control larvae were free of bacterial infection.

3. Toxicity of spent medium produced by selected isolates of protoplasts of *Entomophthora egressa*. Washed protoplasts produced from the conidia or mycelia of I458 and I521 and not produce a mycosis or signs of paralysis in the larvae.

The spent medium, regardless of the source of protoplasts, produced equal levels of total paralysis in the larvae at levels greater than the control injections (Table XXXII).



Table XXXI  
Effects of MGM<sup>a</sup> plus protoplasts and spent MGM  
on the mobility of sixth instar spruce budworm

Treatment	Time <sup>b</sup> (hours)	Percentage of larvae with:		
		Paralysis	Partial paralysis	Septicemia
Spent MGM (6 $\mu$ l)	2	100.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spent MGM (3 $\mu$ l) D <sup>c</sup>		100.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spent MGM + protoplasts		100.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
MGM-6 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
MGM-3 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spent MGM (6 $\mu$ l)	18	85.7 $\pm$ 2.3	14.3 $\pm$ 3.5	11.1 $\pm$ 1.1
Spent MGM (3 $\mu$ l) D		77.8 $\pm$ 5.2	22.2 $\pm$ 2.7	0.0 $\pm$ 0.0
Spent MGM + protoplasts		80.1 $\pm$ 7.3	19.9 $\pm$ 2.9	0.0 $\pm$ 0.0
MGM-6 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
MGM-3 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spent MGM (6 $\mu$ l)	42	77.4 $\pm$ 2.6	22.6 $\pm$ 1.8	28.2 $\pm$ 1.6
Spent MGM (3 $\mu$ l)		71.8 $\pm$ 2.1	28.2 $\pm$ 3.1	11.2 $\pm$ 1.1
Spent MGM + protoplasts		72.9 $\pm$ 1.3	27.1 $\pm$ 1.9	12.7 $\pm$ 1.8

cont.

Table XXXI cont.

Treatment	Time (hours)	Percentage of larvae with:		
		Paralysis	Partial paralysis	Septicemia
MGM-6 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
MGM-3 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spent MGM (6 $\mu$ l)	76	70.1 $\pm$ 2.1	29.9 $\pm$ 1.7	34.7 $\pm$ 1.1
Spent MGM (3 $\mu$ l) D		72.3 $\pm$ 2.0	27.7 $\pm$ 1.8	27.2 $\pm$ 0.9
Spent MGM + protoplasts		71.9 $\pm$ 1.3	28.1 $\pm$ 2.1	22.9 $\pm$ 1.9
MGM-6 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
MGM-3 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Spent MGM (6 $\mu$ l)	102	70.1 $\pm$ 2.1	29.9 $\pm$ 1.7	38.2 $\pm$ 2.1
Spent MGM (3 $\mu$ l) D		72.1 $\pm$ 2.2	27.9 $\pm$ 1.6	37.1 $\pm$ 2.7
Spent MGM + protoplasts		71.9 $\pm$ 1.3	28.1 $\pm$ 2.1	35.3 $\pm$ 1.9
MGM-6 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
MGM-3 $\mu$ l-control		0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

<sup>a</sup>Modified Grace's insect tissue culture medium<sup>b</sup>Time post injection<sup>c</sup>Diluted 10<sup>-4</sup>

(Note: Only sixth instar female larvae used)



Table XXXII

Level of total paralysis of sixth instar  
spruce budworm injected with spent MGM  
previously containing selected isolates of  
the protoplasts of Entomophthora egressa

Isolate	Protoplast source	Total paralysis (%) <sup>a</sup>
521	Conidia	99.0 $\pm$ 1.2
	Hyphae	98.2 $\pm$ 1.7
458	Conidia	100.0 $\pm$ 0.9
	Hyphae	99.8 $\pm$ 1.2
	MGM <sup>b</sup> control	0.0 $\pm$ 0.0

<sup>a</sup>42h post injection of 3 l medium diluted  $10^{-4}$

<sup>b</sup>Modified Grace's medium

(Note: Only sixth instar female larvae used)

4. Centrifugation effects on selected protoplast isolates. The failure of protoplasts to develop in the spruce budworm larvae may have reflected centrifuge-induced damage to the former. It was necessary to determine the effects of centrifugation at various gravities on the protoplasts of both isolates.

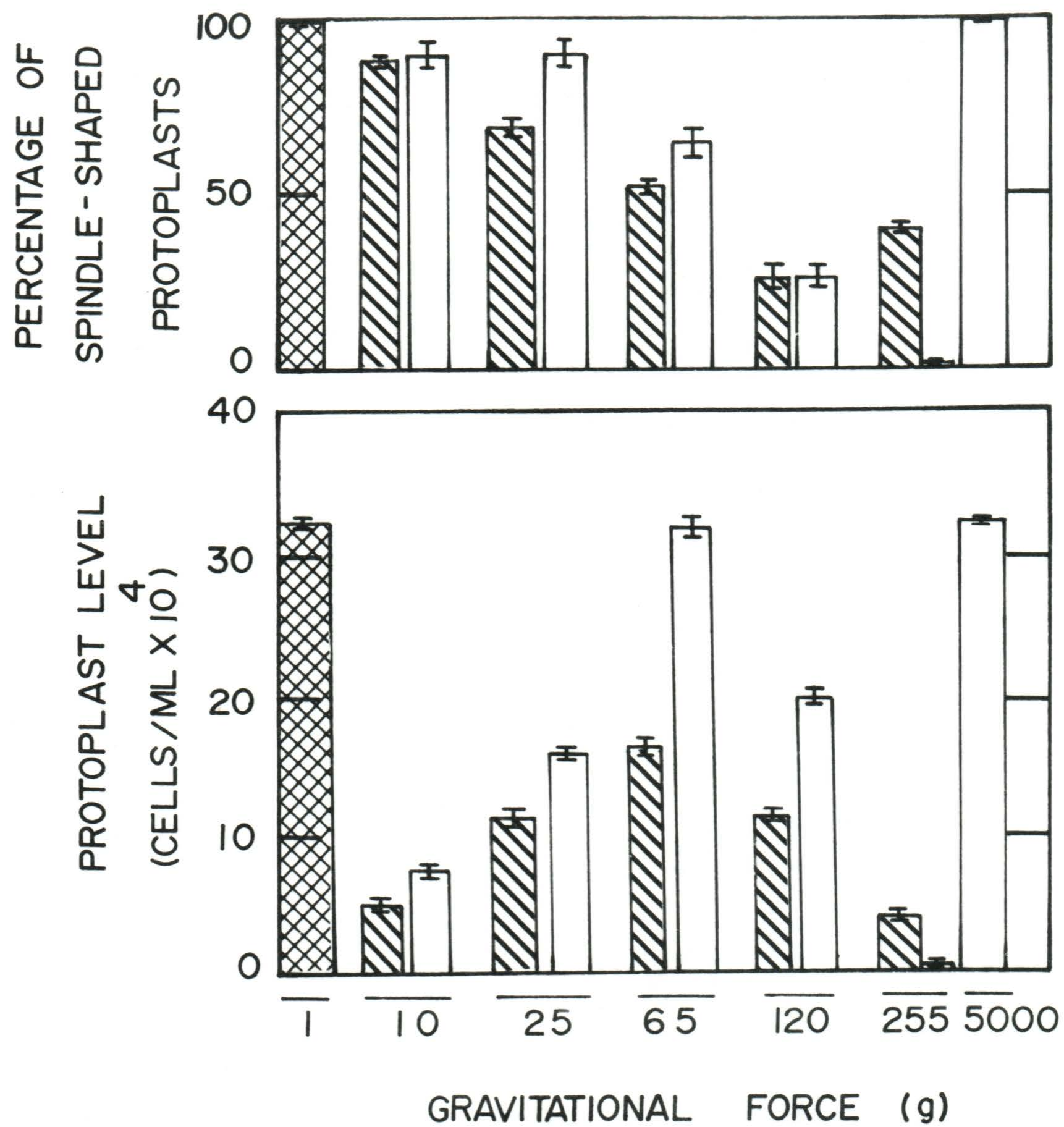
Centrifuging protoplasts from the conidia of E. egressa of I521 revealed that both the time of centrifugation and the force of centrifugation influenced the percentage of spindle-shaped protoplasts and the level of protoplasts. For a given time, with increasing centrifugal force, the numbers of spindle-shaped cells declined (Fig. 51). The effects were more pronounced with protoplasts centrifuged for 5min. In all cases the levels were less than the control and regularly centrifuge-washed cells (5000xg) (Fig. 51).

The level of protoplasts/ml increased with increasing centrifugal force up to 65xg for a given time and declined thereafter (Fig. 52). Increasing the time of centrifugation for a given centrifugal force also increased the protoplast level (Fig. 52). The level at 65xg for 7min was equal to that of regularly centrifuge-washed cells. The effects of centrifugation on protoplast levels were more pronounced with cells collected after 7min centrifugation.



Fig. 51. Percentage of the spindle-shaped protoplasts of Entomophthora egressa isolate 521 from conidia after centrifugation at various gravitational forces for 5min (▧) and 7min (| |). Pooled 1xg controls (▧) and pooled 5000xg samples (| |).

Fig. 52. Number of protoplasts of Entomophthora egressa isolate 521 after centrifugation at selected gravitational forces for 5min (▧) and 7min (| |). Pooled 1xg controls (▧) and pooled 5000xg samples (| |).





The patterns were the same for protoplasts from the mycelium and conidia of I458 (Figs. 53 and 54). The levels of spindle-shaped protoplasts and the levels of collected protoplasts of I521 were greater than the results of I458.

Protoplasts from the conidia of I521 were analysed for the effects of centrifugation on protoplast growth rates.

The early growth profiles of protoplasts exposed to 10xg were highly aberrant with lysis occurring at 22.5h and 18.5h for cells so treated for 5 and 7min, respectively (Fig. 55). The population of protoplasts after centrifugation at 25xg was devoid of cellular lysis for both time regimes (Fig. 56).

The growth rate of the 5min treated cells was equal to the growth rate of the 7min treated cells at 6h/generation and 5h/generation, respectively. The 7min treated cells were slower in the early growth period than the 5min treated protoplasts (Fig. 56). The final yield was greatest for protoplasts centrifuged for 7min and equal to the control value (Fig. 56).

Protoplasts centrifuged at 65xg for 5min revealed a lytic period at 26.5h which was not detected for protoplasts centrifuged at 65xg for 7min (Fig. 57). The latter proto-

Fig. 53. Pooled level of spindle-shaped protoplasts of Entomophthora egressa isolate 458 from conidia and mycelium after exposure to selected gravitational forces for 5min (▨) and 7min (||). Pooled 1xg controls (▩) and 5000xg (||) samples.

Fig. 54. Pooled level of protoplasts of Entomophthora egressa isolate 458 from conidia and mycelium after exposure to selected gravitational forces for 5min (▨) and 7min (||). Pooled 1xg controls (▩) and 5000xg samples (||).



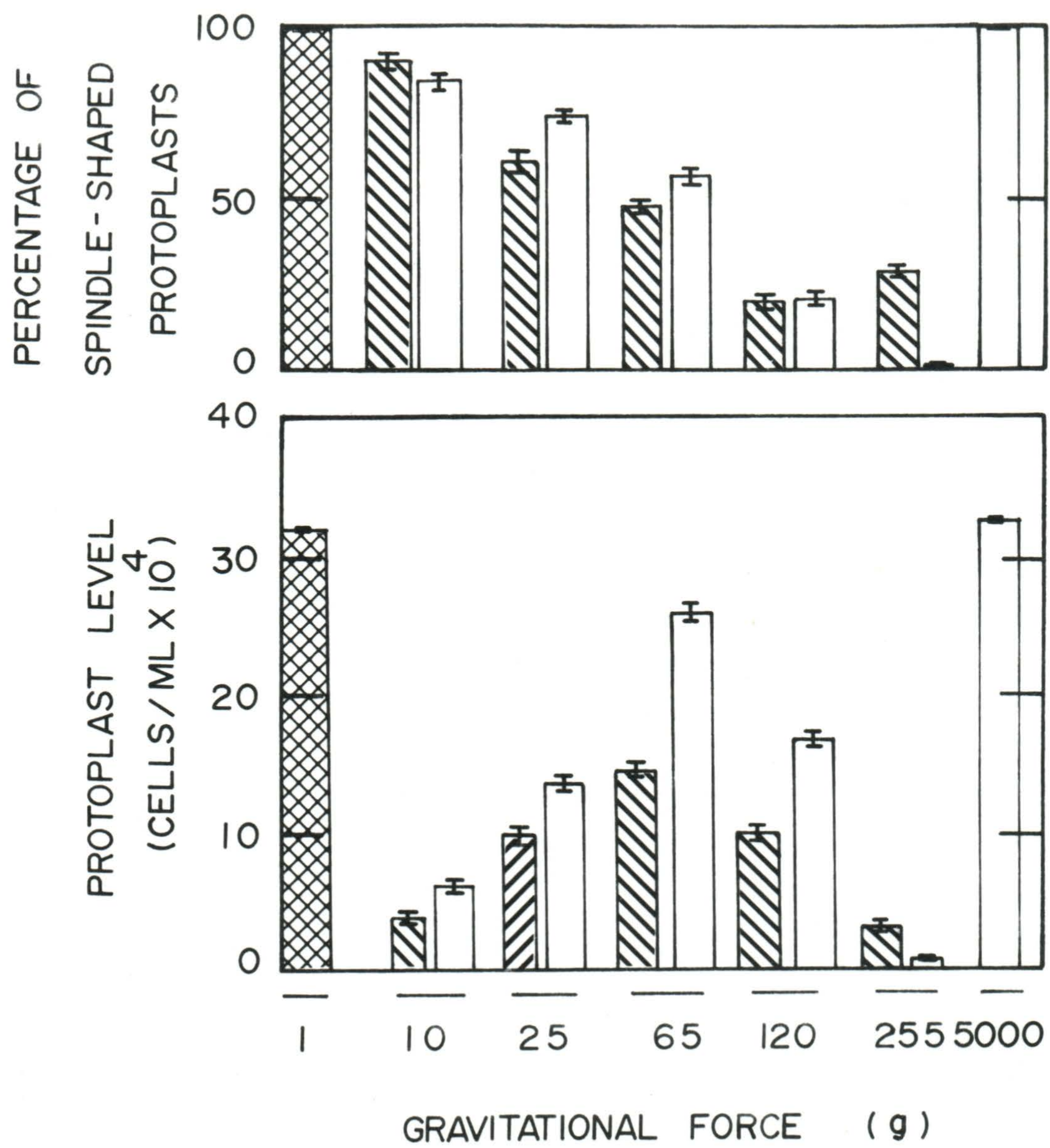


Fig. 55. Effects of centrifugation at 10xg for 5 min (○) and 7min (●) on the growth of protoplasts of isolate 521 of Entomophthora egressa from conidia.

Fig. 56. Effects of centrifugation at 25xg for 5min (○) and 7min (●) on the growth of protoplasts of isolate 521 of Entomophthora egressa from conidia. Control growth profile (▲).



PROTOPLAST CONCENTRATION (CELLS/ML  $\times 10^3$ )

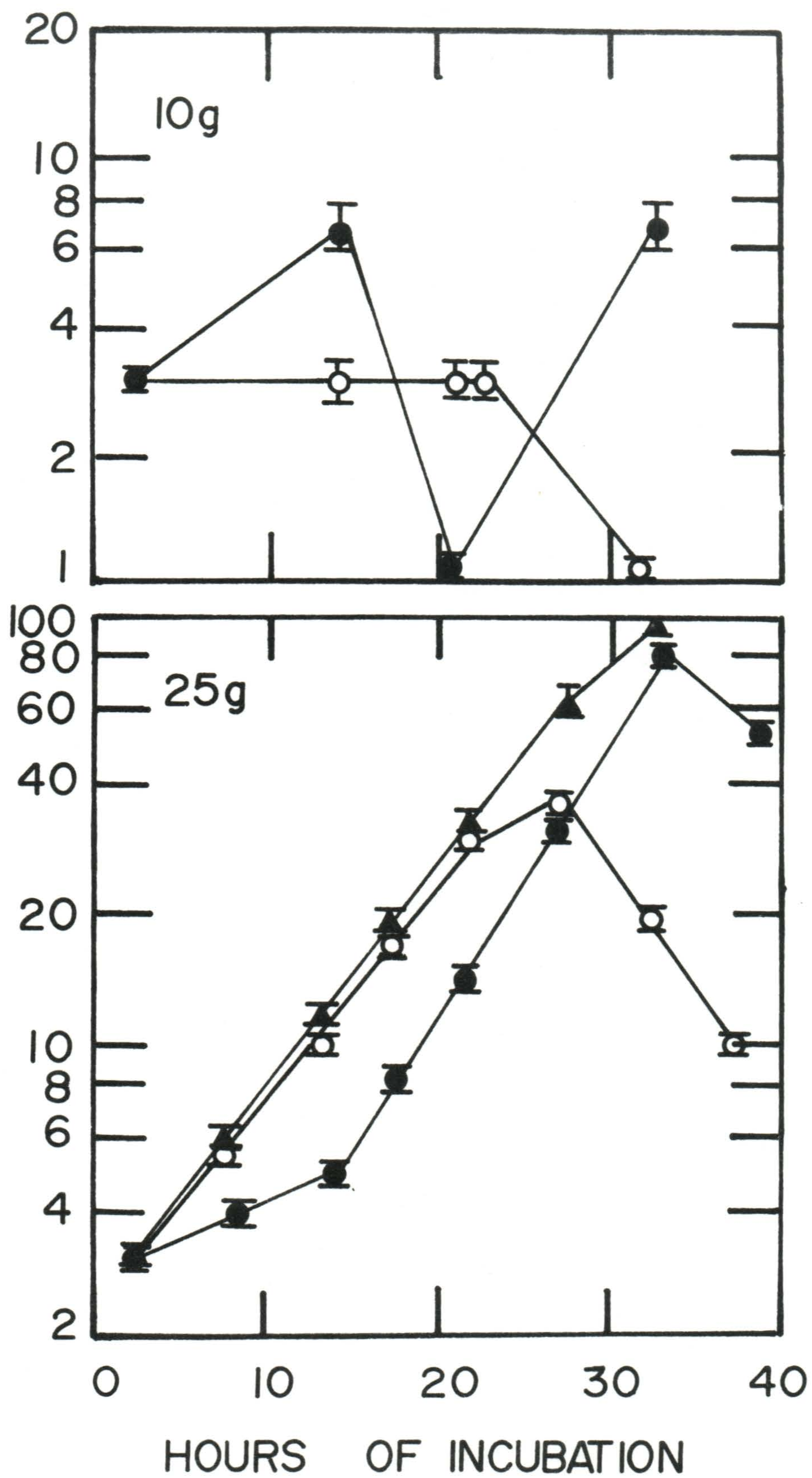
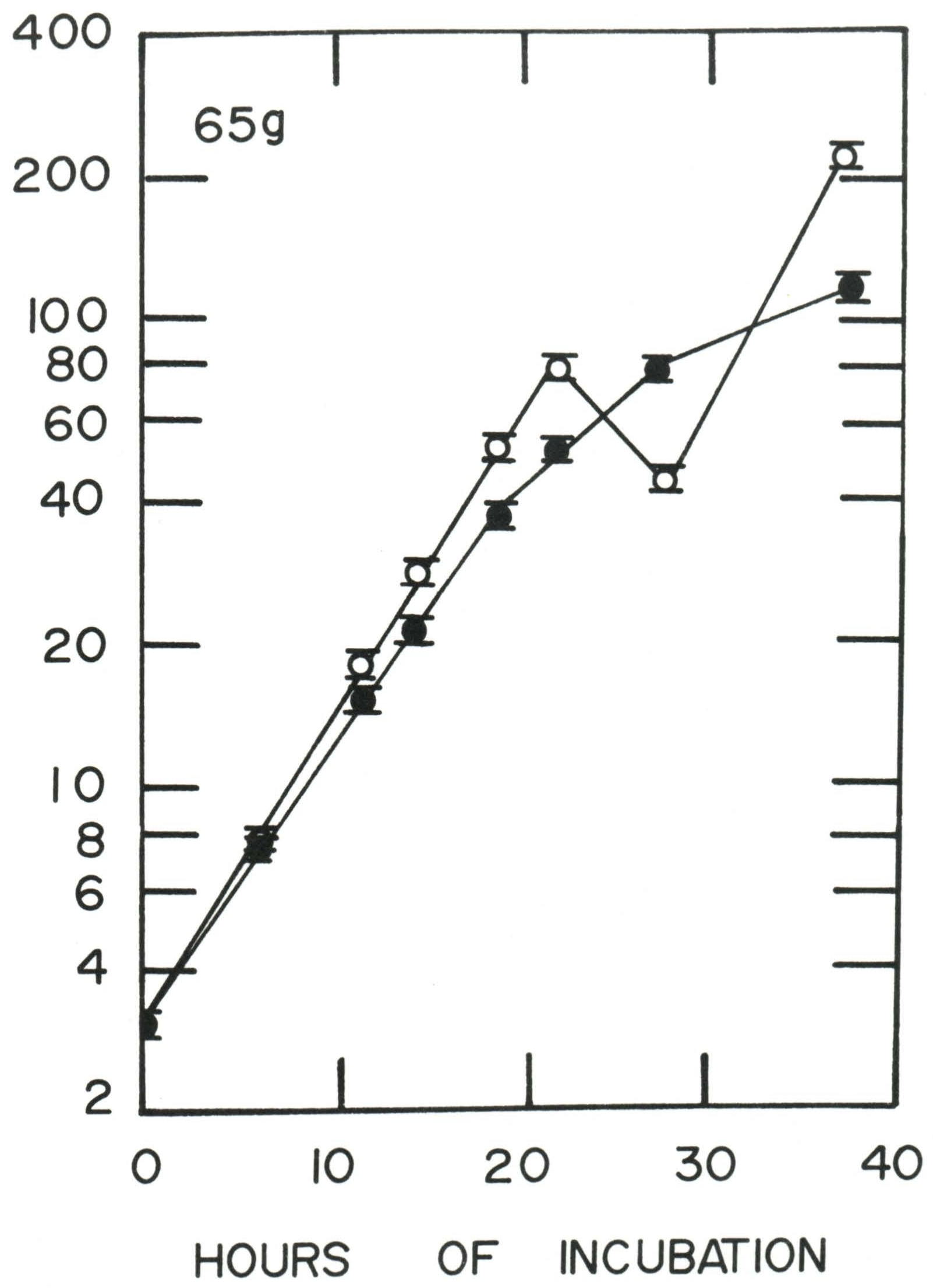


Fig. 57. Effects of centrifugation at 65xg for 5min (○) and 7min (●) on the growth of protoplasts of isolate 521 of Entomophthora egressa from conidia.



PROTOPLAST CONCENTRATION (CELLS/ML  $\times 10^3$ )



plasts grew more slowly than the former with 6h/generation and 4h/generation, respectively. Unlike the profiles of the previous protoplast groups the 65xg protoplasts, after 7min centrifugation grew at a rate comparable to the control protoplasts (Fig. 56).

At centrifugal forces greater than 65xg sporadic protoplast growth and lysis was exhibited (Figs. 58 and 59).

Because protoplasts produced from the conidia of I521 were resistant to lysis at 65xg such treated protoplasts were injected into spruce budworm larvae. Again the larvae failed to develop a fungal infection.

5. Effects of mycotoxin containing medium on the DHC profile. Spent medium did not change the levels of the prohemocytes, plasmatocytes, granular cells or spherule cells compared to the MGM-injected control larvae (Table XXXIII) 1h postinjection. The level of oenocytoids declined in larvae receiving spent MGM.

By 18h many of the hemocytes had extensive cytoplasmic vacuolation. The spherule cells discharged the spherules during observation. Within 24h post injection the hemolymph was full of cellular debris and motile rod-shaped bacteria. The few remaining hemocytes were highly vacuolated.



Fig. 58. Effects of centrifugation at 120xg for 5min (○) and 7min (●) on the growth of protoplasts of isolate 521 of Entomophthora egressa from conidia.

PROTOPLAST CONCENTRATION (CELLS/ML X 10<sup>3</sup>)

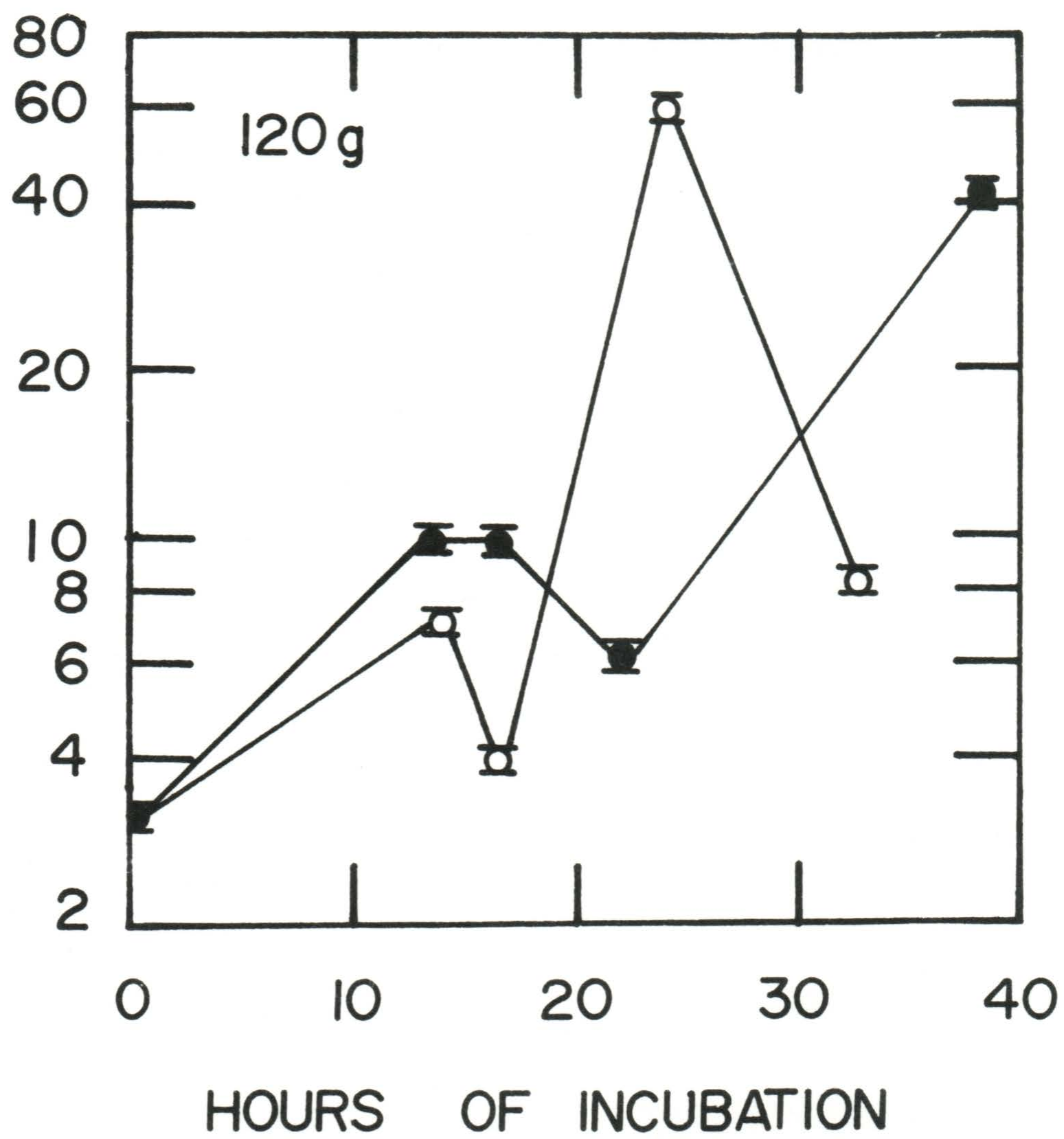




Fig. 59. Effects of centrifugation at 225xg for 5min (O) on the growth of protoplasts of isolate 521 of Entomophthora egressa from conidia.

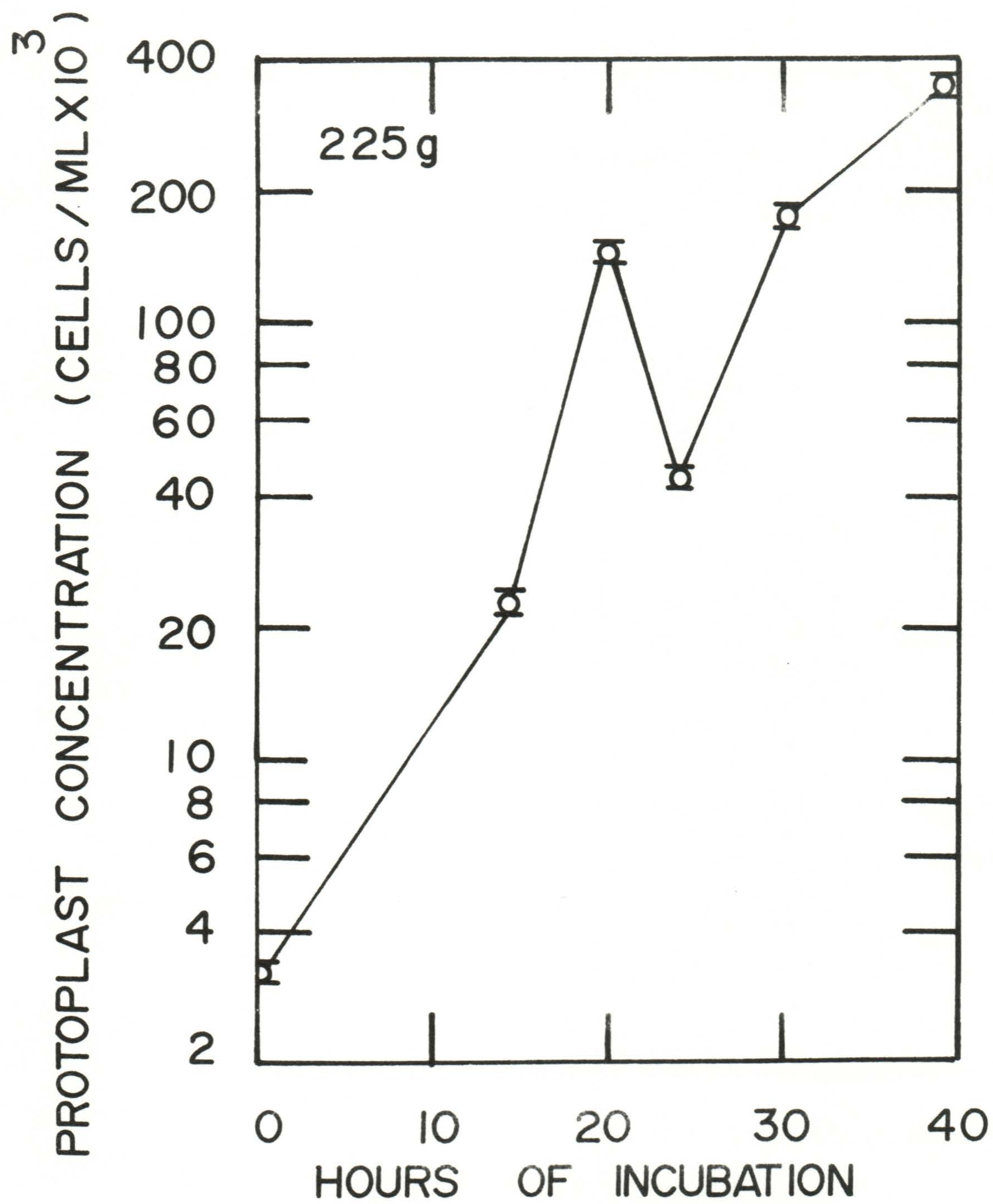




Table XXXIII  
Effect of mycotoxin containing medium<sup>a</sup> on the  
differential hemocyte count<sup>b</sup> of female sixth instar  
female spruce budworm larvae 18h after injection

Treatment	Pr <sup>c</sup>	Pl	Gr	Sp	Oe
MGM control	2.6 ± 1.0 <sup>d</sup>	9.4 ± 3.5	78.4 ± 3.1	8.5 ± 1.4	2.0 ± 0.9
Spent MGM	1.3 ± 0.5	18.7 ± 4.6	79.3 ± 2.7	5.2 ± 1.5	0.0 ± 0.0
T-value	1.163	1.609	0.219	1.608	2.222
(P-value)	(P>0.2)	(P>0.1)	(P>0.6)	(P>0.1)	(P<0.05)

<sup>a</sup>3ul injection of spent MGM diluted 10<sup>-4</sup>

<sup>b</sup>1h post injection

<sup>c</sup>Pr-prohemocyte, Pl-plasmatocyte, Gr-granular cell, Sp-spherule cells  
Oe-oenocytoid

<sup>d</sup>Sample size of 23 larvae, 100 hemocyte examined/larva

6. Toxin production studies. In an attempt to elucidate the nature of the toxin(s) the ability of spent medium to induce partial larval paralysis was correlated with changes in medium pH, osmolality, total protein level and protoplast concentration.

Fifty percent of the larvae receiving 20h old spent MGM were permanently partially paralysed (Fig. 60). By 40h of protoplast growth, the level of partial paralysis was 100%. Total paralysis occurred in 20% of the larvae receiving medium at the peak of protoplast growth. The level of total paralysis declined during protoplast regeneration, but the level of partial paralysis was constant. The level of partial paralysis appeared to be independent of any change in medium pH, osmolality or total protein level (Fig. 60). The latter two parameters could not be totally discounted.

7. Osmolality studies. Although graphic analysis suggested that changes in osmolality were not correlated with the induction of larval paralysis, the possibility of a contribution to paralysis was not disproven.

Both carbohydrates (sucrose and trehalose) induced partial paralysis at the osmolalities tested (Table XXXIV). With increasing concentration and osmolality, the level of partial paralysis increased. Sucrose was less effective



Fig. 60. Production of a paralysis-inducing toxin by protoplasts of Entomophthora egressa isolate 521. pH (●), protoplast levels (○), level of partial paralysis (◐), total protein level (▲) and osmolality (△).

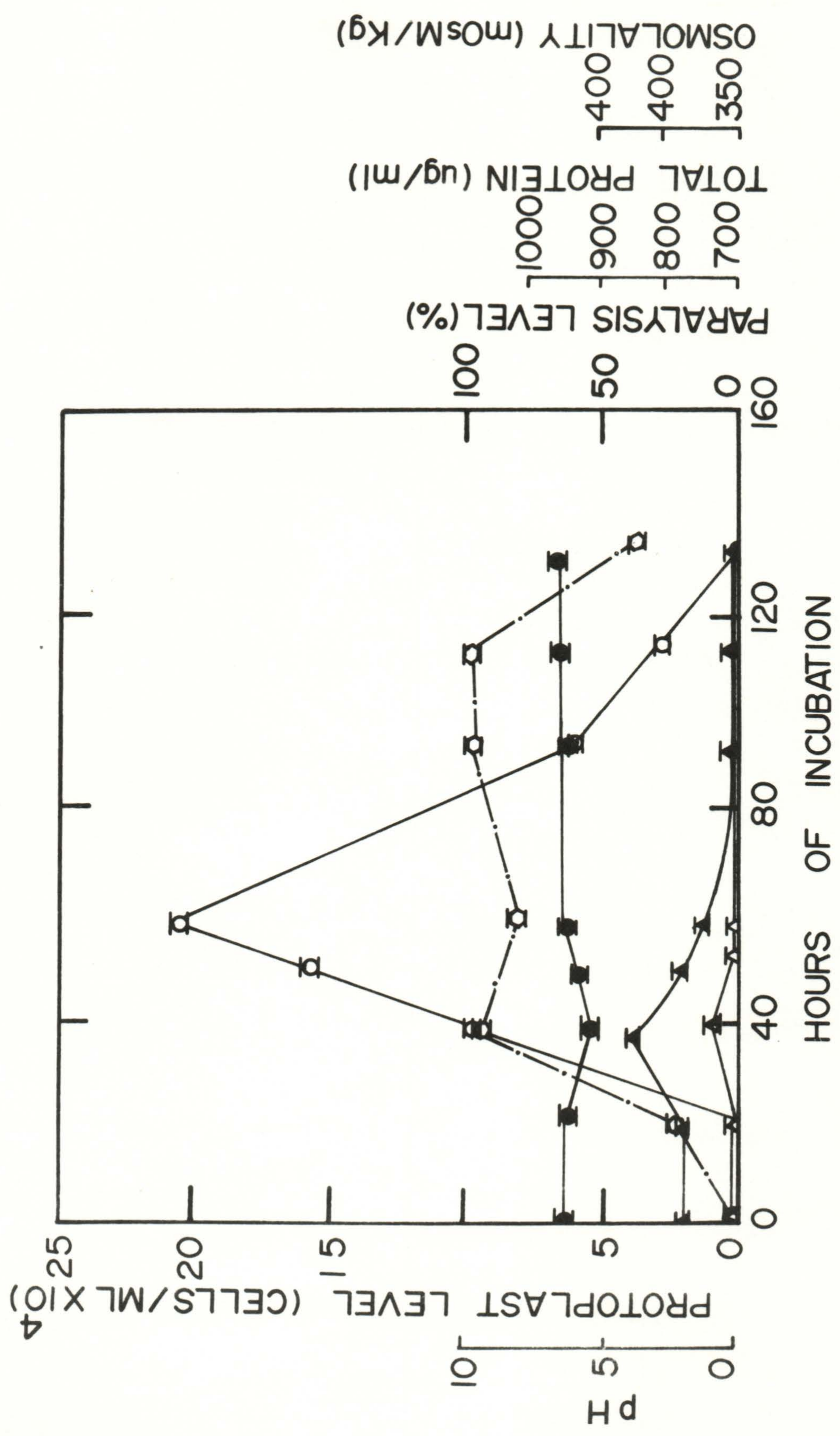




Table XXXIV  
Effects of trehalose and sucrose solutions<sup>a</sup>  
at selected osmolalities on the incidence of  
partial paralysis of sixth instar spruce budworm

Carbohydrate used	Osmolality of solution	Percentage of larvae paralysed		
		0.5h	2h	24h
Trehalose	300	33.3 ± 1.2	21.2 ± 0.9	2.1 ± 1.1
	350	38.2 ± 2.7	23.1 ± 1.1	1.7 ± 1.0
	570	67.3 ± 1.9	25.2 ± 1.0	3.7 ± 1.8
	800	99.5 ± 1.0	61.1 ± 2.3	4.3 ± 2.2
Sucrose	300	31.7 ± 2.9	20.8 ± 1.0	1.7 ± 0.8
	350	37.8 ± 2.8	17.9 ± 2.1	1.5 ± 0.7
	570	58.9 ± 2.3	27.2 ± 1.1	1.8 ± 1.0
	800	83.9 ± 2.8	29.5 ± 1.0	1.7 ± 0.9
MGM control	360	1.2 ± 1.0	0.0 ± 0.0	0.0 ± 0.0

<sup>a</sup>3ul injections

(Note: Only sixth instar female larvae used)

in causing this response than trehalose (Table XXXIV). The effects diminished with time; and after 24h, all larvae were capable of normal movement.

8. Effects of temperature and pH on the toxicity of spent MGM. The toxin was moderately heat stable because the medium's paralytic property was maintained after heating for 15 and 30min at 65°C and 75°C (Table XXXV). Heating at either of these temperatures for 60min lowered the level of larval paralysis.

Trichloroacetic acid lowered the toxicity of spent MGM to the levels of the MGM + TCA and MGM control groups ( $F=0.532$ ,  $P>0.4$  (Table XXXV)).



Table XXXV  
Effects of temperature and pH on  
the induction of larval paralysis by spent MGM<sup>a</sup>

Treatment	Level of total paralysis (%)	t-value (P-value)
65°C for 15min	98.7 ± 1.8	
65°C for 30min	98.9 ± 2.1	0.072 (P>0.900) <sup>b</sup>
65°C for 60min	76.2 ± 1.7	9.088 (P<0.001)
75°C for 15min	100.0 ± 0.7	
75°C for 30min	98.8 ± 1.1	1.534 (P>0.100)
75°C for 60min	53.2 ± 1.3	31.696 (P<0.001)
Spent MGM	92.8 ± 4.9	
Spent MGM + TCA <sup>c</sup>	2.0 ± 0.9	76.059 (P<0.001) <sup>d</sup>
MGM control	1.7 ± 0.1	107.234 (P<0.001)
MGM control + TCA	1.9 ± 0.1	108.197 (P<0.001)

<sup>a</sup>Modified Grace's insect tissue culture medium

<sup>b</sup>Comparison between 15min and the other times for a given temperature at which spent MGM was heated

<sup>c</sup>Trichloroacetic acid

<sup>d</sup>Comparison between Spent MGM and other treatments

(Note: Only sixth instar female larvae used)



B. Discussion. Spent MGM contained a potent paralysis-inducing factor, which by its rapid action at high dilutions, in the absence of a bloated larval body, would appear to be a neurotoxin. This ability was not isolate variable. Insect paralytic mycotoxins in spent culture media have been previously reported. Culture media of A. flavus and B. bassiana have been reported to induce an ephemeral paralysis in the nymphs and adults of Locusta migratoria manilensis Mey shortly after injection (Evlakhov and Ratkinin 1963). The recovered nymphs did not metamorphose into adults. Roberts (1966a,b) observed tetanic paralysis in B. mori larvae infected with M. anisopliae. Beauveria bassiana, P. farinosus and P. fumoso-roseus produced paralytic factors against G. mellonella (Wojciechowska 1973). Paralysis occurred at a much slower rate than in spruce budworm larvae receiving E. egressa spent medium.

The toxin(s) of the present study did not alter the hemogram of the larvae but later induced extensive cytoplasmic vacuolation and hemocyte degeneration. Prasertphon and Tanada (1969) reported that the injection of culture filtrates of E. coronata or E. apiculata containing mycotoxin, into G. mellonella, H. zea, H. cecropia and B. mori induced hemocyte clumping. This was not observed in the present study. Injection of exotoxins of Bacillus thuringiensis Berliner into L. migratoria migratorioides has been reported



to induce pathological changes in hemocyte structure but not to alter the hemogram (Hoffmann et al. 1974). Belloncik and Charbi-Said (1977) have reported that toxins from C. *militaris* destroy the cellular integrity of cells of Aedes *albopictus* (Skuse) Singh in vitro. Feir (1979) has proposed that the data on hemocyte-toxin interaction is highly conclusive.

The decline in hemocyte levels attributed to their degeneration favoured septicemia. The source of the bacteria was not determined. The toxin(s) did not have any antibacterial activity. Antibacterial activity has been reported for mycotoxins of E. *coronata* and E. *apiculata* (Prasertphon and Tanada 1968).

The absence of mycoses caused by selected strains of I521 remains puzzling. Perhaps the damage occurred by the protoplasts during centrifugation, while not affecting the absence of protoplast-hemocyte interaction in vitro may have made the protoplasts more susceptible to resistance mechanisms in vivo. Differences between the source of protoplasts within and between isolates substantiates the isolate differences documented in section IV. A. and B.

The production of the paralytic factor(s) was correlated with protoplast growth but not with medium pH. Wojciechowska (1973) documented paralytic toxin production and its corre-



lation with the growth of B. bassiana, P. farinosus and P. fumoso-roseus. The decline in spruce budworm total paralysis suggested that either the toxin(s) was unstable or that the protoplasts removed the toxin(s) from the medium. Spent medium devoid of protoplasts lost the paralytic ability after 72h at 4°C. Prasertphon and Tanada (1969) reported that the pH of culture filtrates was unrelated to the toxicity of the media.

If osmotic changes in the medium containing protoplasts of E. egressa were responsible, in part, for larval paralysis, the evidence using sucrose and trehalose solutions suggests the influence would be short-term.

The major paralytic factor(s) in spent MGM was a heat labile protein. Protein mycotoxins have been produced by E. coronata and E. apiculata (Prasertphon 1968, Prasertphon and Tanada 1969). Unlike the toxins of the latter two species, the toxin of E. egressa did not induce larval discoloration. On the basis of the symptoms, it would appear that the mycotoxin of E. egressa differed from those of E. coronata and E. apiculata.



### Summary

It is the wall-free protoplast stage of I458 and I521 of E. egressa which exists in the hemocoels of the eastern hemlock looper larvae and eastern spruce budworm larvae, respectively. Initial work by Tyrrell (1977) suggested that the spruce budworm hemocytes did not physically attach to the protoplasts of several isolates of E. egressa in vivo.

The results of the present thesis establish the absence of hemocyte adhesion to protoplasts of either isolate of E. egressa in their proper hosts. There was no hemocyte-protoplast adhesion in the sixth instar female spruce budworm larvae containing I458 of E. egressa.

In terms of the host species this may reflect the absence of an immune response to nonself materials. To test this hypothesis both species of insect were exposed to E. coli, B. cereus, spores of A. repens and hyphae of R. nigricans. In all cases the granular cells of the five types of hemocytes detected in both insect species (prohemocytes, plasmatocytes, granular cells, spherule cells and oenocytoids) were involved with contacting and adhering to test particles. Both the hemlock looper and spruce budworm granular cells revealed aspects of nodulation with the bacteria. When spores of A. repens were used nodulation was not observed. The hyphae of R. nigricans was encapsulated by the



granular cells of the hemlock looper in vitro. Exposure of the Shb and hyphae of I458 of E. egressa to hemlock looper hemocytes resulted in the adhesion of the spherule cells to the I458 walled stages. The response of a given species of insect was, therefore, a function of the type of test particles used. Spruce budworm larval hemocytes did not adhere to the Shb, germinating Shb or rod-shaped hyphal bodies of E. egressa I458 or I521 in vitro. Thus, the response to a given type of test particle varied with the species of insect.

Because of supply problems with the hemlock looper the sixth instar female spruce budworm larvae were used for the remainder of the study.

The host larvae were capable of responding to nonself materials other than the protoplasts. The absence of hemocytes adhering to E. egressa protoplasts may be due to the following:

(i) the protoplasts may be secreting a metabolite suppressing hemocyte activity

(ii) the protoplasts may have acquired larval plasma molecules about the surface masking recognition by the hemocytes

or (iii) the protoplasts may have a unique, innate coating preventing hemocyte adhesion.



The suppression of hemocyte activity would be a detrimental property of the mycosis because it would render the host insect more susceptible to disease and by extension reduce the probability of the development of a successful mycosis due to competition between the protoplasts and other pathogens. There was no evidence of the granular cells of either the hemlock looper or spruce budworm larvae being inhibited by protoplasts of I458. Isolate 521 protoplasts did not inhibit the adhesion of spruce budworm granulocytes to the hyphae of either I458 or I521.

Exposing protoplasts of E. egressa I521 or I458 centrifuge-washed in serum-free MGM to the hemocytes of the spruce budworm also centrifuge-washed in serum-free MGM failed to reveal hemocyte adhesion to the protoplasts. The protoplasts are not believed to have acquired protection from the host hemocytes by the acquisition of host serum molecules.

In the overall the evidence lead to the belief that the surfaces of the protoplasts were responsible for the negative hemocyte response to I458 and I521 protoplasts. Trypsinizing the protoplasts of E. egressa I521 did not enhance the adhesion of the test particles to the protoplasts. Protoplasts of I458 and I521 exposed to papain were readily attacked by the granular cells of the spruce budworm larvae. In some unknown fashion the proteins on the surfaces of both



protoplast isolates protected the protoplasts of hemocyte attack. Based on the ability of selected carbohydrates to enhance or suppress the adhesion of A. repens spores to spruce budworm granular cells one may speculate that glycoproteins may be involved in protecting the protoplast isolates.

Both I458 and I521 protoplasts were able to develop into a conidiophore bearing mycelium in female sixth instar spruce budworm larvae. This does not mean that both isolates are equally adapted to a given host. To test this hypothesis the ideal situation would be to study in vivo the development of both isolates in the host hemocoel. Because of the presence of a potent proteinaceous mycotoxin(s) this was not possible. Attempts to wash the protoplasts free of the mycotoxin(s) damaged the cells such that an insect mycosis was not feasible.

In an attempt to speculate on the level of different degrees adaptation by the protoplasts basic physiological properties were compared between both isolates growing in vitro in MGM. Isolate 458 grew faster than I521 and achieved greater cell levels. The regeneration sequences differed between isolates. Isolate 521 protoplasts formed Shb which eventually formed mycelial balls; whereas, I458 protoplasts formed fusion spheres. The differences in metabolism be-



tween the walled stages of I458 and I521 were revealed by analysing changes in total protein, glucose and NPC levels in MGM. The protoplast stage of both isolates, the stage found in the hemocoel of the spruce budworm larvae, also revealed differences with relation to changes induced in MGM during protoplast growth. Several major differences included I458 protoplasts utilizing glucose at a lower rate than I521 protoplasts and the former growing faster than the latter in the presence of elevated levels of fructose and lowered levels of glucose. In view of the glucose utilization rates between isolates it may be that protoplasts of I458 utilized fructose to a greater extent than did I521 protoplasts. Both isolates of protoplasts of E. egressa revealed an optimum CO<sub>2</sub> level for growth of 3%. The growth rates and profiles for a given CO<sub>2</sub> level varied between protoplast isolates. Phytophagous lepidoptera are reported to contain between 2% and 4% CO<sub>2</sub> in their hemolymph (Roeder 1953). How these differences relate to the in vivo situation remains to be determined but they establish the potential for differences in adaptation by the isolates to the host.

A survey of the literature revealed the importance of insect plasma proteins in humoral immunity. Prior to exploring the possibility of protein based responses by spruce budworm larvae it was necessary to analyse larval blood volume, total hemolymph protein levels and the degree of protein band



resolution on polyacrylamide gels for different instars and both sexes. The nature of the serological work required large volumes of blood. The greatest volume of hemolymph was in the female sixth instar larvae. The male larvae at a given instar contained less hemolymph than the female larvae. A study of total hemolymph protein levels revealed the female larvae achieved maximum level by the fifth instar; whereas, the male larvae achieved this level by the late sixth instar. Prolonged rearing reduced the number of larvae available to work with and, by extension, the hemolymph supply. The sixth instar female larvae appeared to be the most likely candidates. Resolution of proteins on the polyacrylamide gel was important. The greater the resolution the greater the probability of detecting changes in electropherograms pending plasma proteins interacting with protoplasts of E. egressa. Differences in electropherograms between sexes and between instars were detected. Sixth instar female larval serum yielded the best resolution. All serological work was done using sixth instar female spruce budworm larvae. Only fresh hemolymph could be used because freezing damaged the proteins and influenced the electropherograms by decreasing band number and resolution.

      Viable and nonviable protoplasts of E. egressa I521 were added to whole hemolymph and larval serum. After a period of incubation a decline in total protein was noted



for all test regimes. Because a decline occurred with non-viable protoplasts followed by a fixed level of total protein it is believed that humoral proteins were adhering to the surfaces of the protoplasts. Electrophoresing these samples revealed changes in band number, Rm position and staining intensities compared to the control groups. Differences in electropherograms were observed between hemolymph and serum samples exposed to either living or nonviable protoplasts of I521. Thus, it is contented that the hemocytes interact with the plasma and/or directly or indirectly with the protoplasts. The significance of these interactions remain to be determined.

### Conclusions

1. Hemograms of selected stages of male and female C. fumiferana were determined.
2. Protoplasts of E. egressa did not adhere to the hemocytes of L. fiscellaria fiscellaria. There was no evidence of protoplasts suppressing hemocyte activity. Escherichia coli and sporangiospores of A. repens adhered to the granular cells. These hemocytes adhered to hyphae of R. nigricans. The spherule cells adhered to the spherical hyphal bodies and hyphae of E. egressa.
3. Protoplasts of E. egressa did not adhere to the hemocytes of C. fumiferana. Bacillus cereus and E. coli adhered to the granular cells and were phagocytosed by both the granular cells and the plasmatocytes. Evidence is presented for the recognition of the protoplasts or their metabolites by the hemocytes. Failure of the hemocytes to attach to the protoplasts was not due to the suppression of hemocyte activity. The protoplasts were able to dissociate from the adhering hemocytes of T. molitor an improper host. Granular cells of C. fumiferana adhered to the hyphae of E. egressa.
4. The granular cells of C. fumiferana were selectively inhibited from interacting with spores of A. repens and R. nigricans by PTU. No humoral opsonin activity was involved. Granular cell adhesiveness for spores of A.



repens was enhanced by N-acetylglucosamine and glucosamine. The simple carbohydrates tested suppressed adhesion.

5. The hemolymph of C. fumiferana larvae contained lytic factors active against P. caudatum and E. gracilis.

6. Isolate 458 and isolate 521 of E. egressa exhibited differences in growth patterns and rates on coagulated egg yolk medium, MGM and modified versions of MGM. Differences in the changes of MGM composition were detected. Different responses to CO<sub>2</sub> levels were also observed.

7. Evidence for the involvement of protein receptors in the adhesion of spores of A. repens to the granular cells of C. fumiferana was presented.

8. Evidence for the possible involvement of proto-plast membrane proteins of E. egressa in inhibiting the adhesion of C. fumiferana granular cells was presented.

9. The hemolymph volume, larval hemolymph protein levels and electropherograms of the hemolymph proteins of spruce budworm larvae were determined for both sexes. Female larvae, depending on the stage of development and age, contained more total protein and more hemolymph than the male larvae. Differences in the protein electropherograms were detected between the sexes.

10. Proteins in female spruce budworm larval hemolymph appeared to adhere to the cell surface of E. egressa proto-plasts.

11. The serum of both male and female spruce budworm

larvae, while containing nutrients favouring protoplast development, may contain factors inhibitory to protoplast development. Melanin and possible toxic levels of ninhydrin-positive compounds were the agents implicated.

12. Spent protoplast growth medium contained heat-stable protein(s) capable of inducing paralysis in C. fumiferana larvae.



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# Appendix A

## Grace's insect tissue culture

Component	mg/L	Component	mg/L
CaCl <sub>2</sub>	750.00	L-Histidine	2500.00
KCl	4100.00	L-Isoleucine	50.00
MgCl <sub>2</sub> ·6H <sub>2</sub> O	2280.00	L-Leucine	75.00
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2780.00	L-lysine HCl	625.00
NaHCO <sub>3</sub>	350.00	L-Methionine	50.00
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	1013.00	L-Phenylalanine	150.00
Fructose	400.00	L-Proline	350.00
-Ketoglutaric acid	370.00	DL-Serine	1100.00
Fumaric acid	55.00	L-Threonine	175.00
Glucose	700.00	L-Tryptophan	100.00
Malic acid	670.00	L-Tyrosine	50.00
D-Succinic acid	60.00	L-Valine	100.00
Sucrose	26680.00	Biotin	0.01
B-Alanine	200.00	D-Capantothenate	0.02
L-Alanine	225.00	Choline chloride	0.20
L-Arginine HCl	700.00	Folic acid	0.02
L-Asparagine	350.00	i-Inositol	0.02
L-Aspartic acid	350.00	Niacin	0.02
L-Cystine	22.00	p-Aminobenzoic acid	0.02
L-Glutamic acid	600.00	Pyridoxine HCl	0.02
L-Glutamine	600.00	Riboflavin	0.02
Glycine	650.00	Thiamin HCl	0.02



# Appendix B

Components of medium A, series B and series C							
Component	Concentration (mg/L)						
	A	B	B1	B2	C	C1	C2
CaCl <sub>2</sub>	1000	750	1000	750	750	750	750
KCl	2240	2240	4100	4100	4100	4100	4100
MgCl <sub>2</sub> ·6H <sub>2</sub> O	2280	2280	2280	2280	2280	2280	2280
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2780	2780	2780	2780	2780	2780	2780
NaHCO <sub>3</sub>	350	350	350	350	350	350	350
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	1013	1013	1013	1013	1013	1013	1013
Fructose	400	400	400	400	400	700	700
α-Ketoglutaric acid	370	370	370	370	370	370	370
Fumaric acid	55	55	55	55	55	55	55
Glucose	700	700	700	700	400	700	400
Malic acid	670	670	670	670	670	670	670
D-Succinic acid	60	60	60	60	60	60	60
Sucrose	32828	32841	32733	32810	32810	32752	32795
L-Asparagine	350						
L-Aspartic acid	350						
L-Cystine	22						
L-Glutamine	600						
L-Glutamic acid	600						
L-Leucine	76						
L-Lysine	624						
L-Methionine	50						
L-Proline	350						
DL-Serine	1100						
L-Threonine	175						
L-Tyrosine	50						
L-Valine	100						

The amino acid components were common to all the above media.

Fetal calf serum 27ml/l